The WD40 Repeat Protein WDR-23 Functions with the CUL4/DDB1 Ubiquitin Ligase To Regulate Nuclear Abundance and Activity of SKN-1 in Caenorhabditis elegans

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The transcription factor SKN-1 protects Caenorhabditis elegans from stress and promotes longevity. SKN-1 is regulated by diverse signals that control metabolism, development, and stress responses, but the mechanisms of regulation and signal integration are unknown. We screened the C. elegans genome for regulators of cytoprotective gene expression and identified a new SKN-1 regulatory pathway. SKN-1 protein levels, nuclear accumulation, and activity are repressed by the WD40 repeat protein WDR-23, which interacts with the CUL-4/DDB-1 ubiquitin ligase to presumably target the transcription factor for proteasomal degradation. WDR-23 regulates SKN-1 target genes downstream from p38 mitogen-activated protein kinase, glycogen synthase kinase 3, and insulin-like receptor pathways, suggesting that phosphorylation of SKN-1 may function to modify its interaction with WDR-23 and/or CUL-4/DDB-1. These findings define the mechanism of SKN-1 accumulation in the cell nucleus and provide a new mechanistic framework for understanding how phosphorylation signals are integrated to regulate stress resistance and longevity.

In response to xenobiologic and oxidative stress, eukaryotic cells activate conserved pathways that increase the expression of phase II detoxification enzymes that scavenge free radicals, synthesize glutathione, and catalyze conjugation reactions that increase xenobiotic solubility and excretion (20). Phase II detoxification plays a central role in preventing age-related diseases, such as cancer and neurodegeneration (34, 39), and in mediating the multidrug resistance of pathogenic fungi, helminthes, and tumor cells (30, 44, 57).

Phase II detoxification in Caenorhabditis elegans is controlled by the transcription factor SKN-1 (1), which promotes stress resistance and longevity (1, 2, 31, 55). In nonstressed animals, SKN-1 is constitutively localized in the nuclei of hypothalamic-like (ASI) neurons, where it is required for life span extension by dietary restriction (5). SKN-1 is absent from the nuclei of other cell types except during exposure to oxidative stress and xenobiotics, which induces its accumulation in intestinal-cell nuclei, where it activates the expression of phase II detoxification genes (1, 2, 15, 27, 55). Despite the central role of SKN-1 in stress resistance and longevity, the mechanisms that control nuclear accumulation of the transcription factor are unknown.

Phosphorylation of SKN-1 by glycogen synthase kinase 3 (GSK-3) inhibits nuclear accumulation (2). Nuclear accumulation is also inhibited by phosphorylation via SGK-1, AKT-1, and AKT-2 kinases downstream from the insulin-like receptor DAF-2 (55). Conversely, accumulation of SKN-1 in the nucleus is promoted by phosphorylation by a p38 mitogen-activated protein kinase (MAPK) cascade (23) and the activities of at least four other protein kinases (31). Phosphorylation of SKN-1 by these diverse kinases allows C. elegans to integrate phase II gene expression with metabolism, development, stress, and aging (55). However, the mechanisms by which phosphorylation alters the nuclear accumulation and activity of SKN-1 are unknown.

Cullins are a large superfamily of highly conserved eukaryotic ubiquitin ligases. CUL4 interacts with damaged DNA binding protein 1 (DDB1) in fungi (42), plants (4), C. elegans (33), and mammals (21). The CUL4/DDB1 complex regulates numerous nuclear processes, such as the DNA damage response, DNA replication, and chromatin remodeling (19, 37). Binding of CUL4/DDB1 to substrates catalyzes selective protein ubiquitylation and subsequent degradation in the proteasome. Recent studies have identified numerous WD40 repeat-containing proteins that interact with CUL4/DDB1 and likely function as substrate recognition subunits (3, 18, 37). However, the substrates of most of these WD40 proteins and the cellular processes in which they function are unknown (37).

To identify the mechanisms of SKN-1 activation and signal integration, we performed a genome-wide RNA interference (RNAi) screen for genes that regulate the transcription of a phase II detoxification gene and defined a pathway that includes the proteasome, DDB-1, CUL-4, and the WD40 repeat protein WDR-23. WDR-23 is expressed in intestinal-, hypodermal-, and neuronal-cell nuclei and interacts with DDB-1 and SKN-1. Loss of function of WDR-23 causes constitutive transcription of phase II detoxification genes, accumulation of SKN-1 in intestinal nuclei, elevation of SKN-1 protein levels, and increased longevity and stress resistance. These findings suggest that SKN-1 constitutively enters the nucleus but is prevented from accumulating by WDR-23, which interacts with the CUL-4/DDB-1 complex and presumably targets the...
transcription factor for proteasomal degradation. Importantly, WDR-23 appears to function downstream from p38 MAPK, GSK-3, and insulin-like receptor kinase, suggesting that phosphorylation of SKN-1 functions to alter its interaction with WDR-23 and/or CUL-4/DDB-1. In summary, our findings define the mechanism of SKN-1 nuclear accumulation and provide a mechanistic framework for understanding how diverse physiological inputs are integrated to regulate SKN-1.

MATERIALS AND METHODS

C. elegans strains. The following strains were used: wild-type N2 Bristol, GR1373 eri-1[mg166]/[CL2166 dsx19[PA15/[Pgst-4::GFP-NLS]]; VP537 err-1[mg286]III; dsx19, VP579 wdr-23[m18177]; dsx19, LD1001 lo007[SKN-1::GFP; rol-6], TJ356 zbx356[DAF-16::GFP; rol-6], CF1038 daf-16(mu86), GR1309 daf-16(mg474[III]; daf-2(e1370)]II, and KU4 sek-1(ksm4). Unless otherwise noted, worms were cultured at 20°C using standard methods (8).

RNAi and genome-wide RNAi screening. RNAi was performed by feeding worms a strain of Escherichia coli [HT115(DE3)] that is engineered to transcribe double-stranded RNA (dsRNA) homologous to a target gene (28). Bacteria with plasmid pPD129.36 were used as a control for nonspecific RNAi effects. This control plasmid expresses 202 bases of dsRNA that are not homologous to any predicted C. elegans gene.

Genome-wide RNAi screening was performed by supplementing the C. elegans ORFeome RNAi feeding library (Open Biosystems, Huntsville, AL) with clones from the original genomic RNAi feeding library (Geneservice, Cambridge, United Kingdom). RNAi was performed as described previously (40) with minor modifications. dsRNA-producing bacteria were grown in Luria-Bertani broth containing a selective antibiotic, washed, and then transferred to 96-well plates. Each well was filled with 50 µl of liquid nematode growth medium (NGM) lacking peptone and containing 0.2% β-lactose (40). Thirty to 50 synchronized VP537 L1 larvae were added to each well. After 3 days, the wells were screened manually for the appearance of Pgst-4::GFP (green fluorescent protein) expression with a Zeiss Stemi SV11 microscope. The RNAi worms were then exposed to 38 µM juglone and rescreened 4 h later for reduced Pgst-4::GFP induction. All genes that gave an RNAi phenotype were rescreened three additional times. Those that scored positive in all three trials were considered regulators of gst-4. The identities of gst-4 regulators were confirmed by sequencing the dsRNA plasmids.

Fluorescence assays. Fluorescence measurements were made using a COPAS Biosort (Union Biometrica, Somerville, MA) on early-adult worms, with similar lengths measured as time of flight. Pgst-4::GFP and DAF-16::GFP images were captured with a Zeiss Stemi SV11 microscope (Chester, VA) fitted with a CCD-camera (Princeton Instruments, Trenton, NJ). Images of SKN-1::GFP were captured with a LSM510 Meta confocal microscope and a Plan-Neofluor 40×/1.3-numerical-aperture oil objective lens (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Quantification of nuclear SKN-1::GFP was performed as described previously (1, 2, 23, 55) using a Plan-Neofluor 10×/0.30-numerical-aperture objective lens.

Yeast two-hybrid assays. Bait and prey plasmids were generated by gateway cloning the reading frames for full-length WDR-23 and DDB-1 into pDEST32 and pDEST22, respectively (Invitrogen, Carlsbad, CA). Interaction was tested on synthetic complete agar that lacked leucine, tryptophan, and histidine and was supplemented with 3-aminoo-2,4-triazole according to the manufacturer’s protocols (ProQuest two-hybrid system; Invitrogen). Novel WDR-23 interactors were identified by sequentially transforming Saccharomyces cerevisiae with bait (pDEST32-WDR-23) and a DNA prey library (Invitrogen). Positive clones were sequenced and tested for self-activation as described previously (9).

A GST–WDR-23 fusion construct was generated by gateway cloning the reading frame of full-length WDR-23 into pDEST 27 (Invitrogen). V5 epitope-tagged SKN-1 was generated by cloning the open reading frame of SKN-1e into pcDNA3.1/V5-DEST (Invitrogen). Cell culture, transfection, lysis, and Western analysis of V5-SKN-1 were carried out as described previously (11).

Quantitative PCR and Western analysis. Quantitative real-time PCR was used to measure mRNA levels as described previously (9). The housekeeping ribosomal protein gene rpl-2 was used as an internal control to calculate relative expression levels by the threshold cycle method. For Western analysis, worms were sonicated in 10 volumes of buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 1% sodium dodecyl sulfate, and protease inhibitor tablet [Roche, Indianapolis, IN]) and centrifuged at 16,100 × g for 5 min. Total protein was measured with a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). Samples were diluted in NuPage LDS buffer (Invitrogen, Carlsbad, CA) and subjected to Western blot analysis using monoclonal anti-SKN-1 antibodies (7). The amount of protein transferred was determined using Ponceau S (Sigma Aldrich, St. Louis, MO) staining. Relative Western blot band and total-protein stain intensities were measured using ImageJ 1.32j software (NIH).

RESULTS

SKN-1 mediates stress-induced transcription of gst-4. Glutathione S-transferases (GSTs) are phase II detoxification enzymes that catalyze the conjugation of glutathione to electrophiles. Expression of the C. elegans gene gst-4 is induced by the redox cycling compound paraquat (54), the naphthoquinone juglone (27), heavy metals (50), and acrylamide (15).

We monitored gst-4 expression using a worm strain expressing a transcriptional GFP reporter (Pgst-4::GFP) (41). Pgst-4::GFP was induced significantly by juglone, hydrogen peroxide, and paraquat. Induction by juglone was more than threefold greater than induction by either paraquat or hydrogen peroxide (Fig. 1). Quinones like juglone generate reactive oxygen species and are also electrophiles that form adducts with diverse macromolecules (56).

To identify novel regulators of gst-4, we performed a genome-wide RNAi screen. Pgst-4::GFP worms were crossed with worms harboring a loss-of-function allele (mg366) of erti-1 that confers RNAi hypersensitivity (32). L1 larvae were fed dsRNA-producing E. coli from a bacterial feeding library covering ~96% of the 19,427 predicted C. elegans genes (28, 51). After 3 days, the worms were screened for constitutive expression of Pgst-4::GFP in the absence of stress. The dsRNA-fed worms were then exposed to 38 µM juglone and rescreened 4 h later for reduced induction of Pgst-4::GFP.

RNAi knockdown of 14 genes, including skn-1, reduced Pgst-4::GFP in the presence of juglone. As shown in Fig. 1, skn-1 RNAi inhibited nearly all juglone-, hydrogen peroxide-, and paraquat-induced Pgst-4::GFP fluorescence, confirming and extending recent findings that SKN-1 mediates the transcription of gst-4 (15, 27).

The proteasome, CUL-4/DDB-1, and the WD40 repeat-containing protein WDR-23 function to suppress gst-4 expression. RNAi of 14 genes consistently induced Pgst-4::GFP fluorescence. As shown in Fig. 2A and B, RNAi of three 19S proteasome subunit-encoding genes, rpn-2, rpn-7, and rpn-8, induced
Pgst-4::GFP expression between two- and fivefold. Knockdown of ddb-1 induced Pgst-4::GFP by over 2.7-fold. ddb-1 encodes the C. elegans orthologue of the mammalian DDB1. DDB1 and CUL4 form a complex that mediates ubiquitinylation of diverse substrates (19, 37). In C. elegans, CUL-4/DDB-1 targets the replication-licensing factor CDT-1 for degradation (33).

C. elegans RNAi screens have predicted false-negative rates of 10 to 30% (52). While CUL-4 was not detected in our screen, the identification of DDB-1 as a regulator of gst-4 transcription suggested that CUL-4 might also play a role. As shown in Fig. 2A and B, feeding worms cul-4 dsRNA-expressing bacteria induced an ~1.3-fold increase in Pgst-4::GFP fluorescence.

The strongest repressor of Pgst-4::GFP identified in our screen was WDR-23, an uncharacterized WD40 repeat-containing protein. Knockdown of wdr-23 induced a nearly 15-fold increase in Pgst-4::GFP fluorescence.

Because our screen was based on transgene expression, we used real-time reverse transcription-PCR to determine if endogenous phase II detoxification gene mRNA levels were also altered by RNAi of the novel gst-4 regulators that we identified. Knockdown of ddb-1, cul-4, and wdr-23 increased gst-4 mRNA levels by 21.6-, 4.6-, and 16.6-fold, respectively.

![Figure 1](http://mcb.asm.org/)

**FIG. 1.** gst-4 transcription is regulated by SKN-1-dependent pathways that are activated by stress. Shown are relative Pgst-4::GFP fluorescence (A) and representative fluorescence micrographs (B) of worms fed control or skn-1 dsRNA and exposed to peroxide (5 mM for 20 min), paraquat (35 mM for 1 h), or juglone (38 μM for 1 h). Fluorescence was measured 6 to 8 h after exposure. Individual data points are plotted to illustrate variability. Means are denoted by lines (n = 48 to 152 worms). All three stressors induced significant (P < 0.001) Pgst-4::GFP fluorescence in control (RNAi) worms. RNAi knockdown of skn-1 significantly (P < 0.01) suppressed Pgst-4::GFP induction by all three stressors.

![Figure 2](http://mcb.asm.org/)

**FIG. 2.** gst-4 transcription is repressed by the proteasome, DDB-1, CUL-4, and WDR-23. Shown are relative Pgst-4::GFP fluorescence (A) and representative fluorescence micrographs (B) of worms fed bacteria producing dsRNA to proteasome components (rpn-2, rpn-7, and rpn-8), ddb-1, cul-4, or wdr-23. RNAi or juglone exposure (Fig. 1) induced Pgst-4::GFP expression in multiple tissues. The strongest induction was observed in the intestine. (A) Individual data points are plotted to illustrate variability. Means are denoted by lines (n = 206 to 649 worms) and were all significantly (P < 0.001) different from a normalized value of 1.0 for control dsRNA-fed worms. (C) Relative gst-4 and gst-30 mRNA levels in worms fed bacteria producing dsRNA to ddb-1, cul-4, or wdr-23. The values are means plus standard errors (n = 4 populations of 20 to 30 worms). *, P < 0.05; **, P < 0.01; and ***, P < 0.001 compared to a normalized value of 1.0 for control dsRNA-fed worms.
We also tested gst-30, encoding a second GST (15) that contains three SKN-1-binding motifs in its 5′ regulatory region. Knockdown of ddb-1, cul-4, and wdr-23 increased gst-30 mRNA expression by 10.2-, 1.8-, and 163-fold, respectively (Fig. 2C). Taken together, these results demonstrate that the proteasome, CUL-4/DDB1, and the WD40 repeat-containing protein WDR-23 suppress expression of phase II detoxification genes.

CUL-4/DDB1 and the proteasome function with WDR-23 in a common pathway. Adaptor proteins interact with CUL4/ DDB1 complexes to mediate substrate specificity for ubiquitinylation and degradation (3, 18, 37). WDR23 is the mammalian orthologue of DDB1, which is predicted to encode a nonfunctional protein (Fig. 4A). Worms homozygous for the tm1817 allele constitutively express high levels of Pgst-4::GFP (Fig. 4B) (18- ± 0.3-fold induction relative to the wild type; P < 0.0001) similar to that observed in animals fed wdr-23 dsRNA (Fig. 2A and B). No significant (P > 0.05) additive induction of Pgst-4::GFP expression was observed in wdr-23 deletion mutants fed bacteria producing dsRNA for rpm-2, rpm-7, rpm-8, or cul-4 (Fig. 4B), suggesting that the proteasome and CUL-4 repress gst-4 expression exclusively through pathways that require WDR-23.

RNAi knockdown of ddb-1 caused a small (~13%) but statistically significant (P < 0.001) increase in Pgst-4::GFP expression (Fig. 4B). However, the increase was ~7% of that induced by RNAi of ddb-1 in wild-type worms (Fig. 2A). These data suggest that DDB1 represses gst-4 expression predominantly, but perhaps not exclusively, through pathways that require WDR-23.

A possible interpretation of the data shown in Fig. 4 is that deletion of wdr-23 induces maximum gst-4 expression that cannot be increased further by independent signaling pathways. To test this possibility, we fed wdr-23 deletion mutants bacteria producing dsRNA for F28D1.1, which encodes a second WD40 repeat protein identified in our RNAi screen. Pgst-4::GFP expression in these worms increased 1.9- ± 0.02-fold (Fig. 4B) above that induced by loss of WDR-23 function alone. F28D1.1 is a homologue of the yeast protein Utp7, which functions in ribosome biogenesis, kinetochore organization, and chromosome segregation (12, 14, 26). Although the mechanism by which F28D1.1 regulates gst-4 expression is unknown, these results demonstrate that other pathways regulating gst-4 expression exist and that Pgst-4::GFP expression is not saturated in wdr-23 deletion mutants. Importantly, these results further support our hypothesis that the proteasome, DDB1, and CUL-4 regulate gst-4 transcription in a common pathway with WDR-23.

WDR-23 interacts with DDB-1 in nuclei. The majority of the WD40 proteins that interact with CUL4/DDB1, including human WDR23, contain a conserved 16-amino-acid DWD box motif (DDB1-binding and WD40 repeat; also known as WDXR, DxD, and CDW) (3, 18, 37) (Fig. 5A). Mutations in the DWD box disrupt DDB1 binding (3, 17, 38). The DWD box is conserved in C. elegans WDR-23 (Fig. 3B and 5A), suggesting that there may be a direct interaction between DDB1 and WDR-23. Yeast two-hybrid analysis confirmed this interaction (Fig. 5B).

In plants and mammals, the interaction between WD40 repeat proteins and DDB1 is disrupted by mutation of an arginine residue to alanine at position 16 in the DWD box (3, 17, 38). Mutation of this arginine in C. elegans WDR-23 prevents it from interacting with DDB-1 (Fig. 5B).

The DDB1/CUL4 ubiquitin ligase complex has well-defined roles in regulating DNA damage repair, DNA replication, and chromatin modifications in the nuclei of eukaryotes (19, 37). In C. elegans, ddb-1 and cul-4 mRNAs are expressed ubiquitously and DDB1 is localized to cell nuclei (33, 59). The data shown in Fig. 4 and 5 indicate that WDR-23 functionally and physiologically interacts with CUL-4/DDB1 ubiquitin ligase complexes to regulate gst-4 transcription. If this is correct, then WDR-23 should be expressed with DDB-1 in cell nuclei. To determine where WDR-23 is expressed, we generated a transgenic worm strain expressing full-length WDR-23 fused to GFP that was under the control of the wdr-23 promoter. As shown in Fig. 5C, WDR-23::GFP is expressed in nuclei of the hypodermis, intestine, and head neurons.

WDR-23 interacts with and regulates nuclear accumulation of SKN-1. To further define the mechanism by which WDR-23 regulates gst-4 transcription, we performed a yeast two-hybrid screen for novel interacting proteins. The full-length WDR-23 protein was used as bait to screen 6.3 × 10^6 cDNA clones. We isolated 17 strongly positive clones, and all encoded SKN-1 (Fig. 6A). SKN-1 is expressed as three splice variants that differ in the lengths of their amino termini (WormBase; California Institute of Technology, Cold Spring Harbor Laboratory, Washington University—St. Louis, and The Wellcome Trust Sanger Institute). All of the WDR-23-interacting clones included the entire open reading frames of SKN-1c and various lengths of full-length SKN-1a. GST affinity assays confirmed the interaction between WDR-23 and SKN-1c (Fig. 6A).

As described previously (15, 27) and shown in Fig. 1, SKN-1 is required for gst-4 transcription. To determine if SKN-1 mediates gst-4 transcription in wdr-23 deletion mutants, we measured Pgst-4::GFP expression in VP579 worms that were fed bacteria producing control, skn-1, or GFP dsRNA. As shown in Fig. 6B (left), Pgst-4::GFP expression was reduced nearly 80% by skn-1 RNAi, demonstrating that the transcription factor mediates gst-4 transcription downstream from WDR-23. The remaining Pgst-4::GFP expression was largely limited to the pharynx. Similar residual Pgst-4::GFP expression also remained in worms fed GFP dsRNA and may represent RNAi resistance in this tissue (Fig. 6B, right).

The nuclear localization of DDB-1 (33) and WDR-23 (Fig. 5C) and the strong SKN-1/WDR-23 interaction suggest that WDR-23 and the ubiquitin ligase function to regulate SKN-1 accumulation in the nucleus. To test this possibility, we used a transgenic worm strain expressing a region of SKN-1 that includes SKN-1b and SKN-1c fused to GFP (1). Under non-stressed conditions, SKN-1::GFP is undetectable except for the two ASI sensory neurons in the head (1, 5). Exposure to stressors or inhibition of the proteasome causes SKN-1::GFP to accumulate in the nuclei of intestinal cells (1, 27). As shown in...
FIG. 3. WDR-23 is evolutionarily conserved. (A) A phylogenetic tree was constructed using the neighbor-joining method with Poisson correction. BLAST searches were used to find the three closest homologues of WDR-23 in *H. sapiens* (human), *D. melanogaster* (fly), *C. elegans* (worm), *A. thaliana* (plant), and *N. crassa* (fungus). Human and *C. elegans* proteins are listed by their names; all other proteins are listed by their GenBank accession numbers. The *Arabidopsis* protein NP_176316 was the most divergent protein and was used to root the tree. G protein /H9252 polypeptide 3, one of the best characterized WD40-repeat proteins, is included for reference. The scale bar represents the fraction of amino acids replaced per site. (B) ClustalW alignment of *C. elegans* (Ce) full-length WDR-23a and WDR-23b with human (h) WDR23.1 and WDR23.2. Shading indicates conserved amino acids. Seven WD40 repeats are highlighted with solid boxes. The DWD box is highlighted with a dashed box. Asterisks indicate cysteine adjacent to a basic amino acid residue.
Fig. 6C and D, RNAi of wdr-23, ddb-1, and cul-4 caused accumulation of SKN-1::GFP in intestinal nuclei.

ASI sensory neurons mediate dietary-restriction-induced longevity in C. elegans. Increased life span requires SKN-1b, which is constitutively localized to the nuclei of these neurons (5). SKN-1b is the shortest SKN-1 variant and includes an exon not present in SKN-1a or SKN-1c. Interestingly, we did not detect SKN-1b in our WDR-23 yeast two-hybrid screen. This suggests that SKN-1b may not interact with WDR-23, which in turn may account for its constitutive localization to ASI nuclei.

Nuclear accumulation of SKN-1::GFP could simply reflect increases in skn-1 mRNA levels. To test this possibility, we performed real-time PCR on wild-type worms that were fed bacteria producing dsRNA for wdr-23, ddb-1, or cul-4. As shown in Fig. 7A, skn-1 mRNA levels were not significantly (P > 0.05) increased by knockdown of these genes, demonstrating that WDR-23 and the CUL-4/DDB-1 complex regulate nuclear accumulation of SKN-1 protein independently of skn-1 mRNA levels.

To determine if nuclear accumulation of SKN-1 reflects increased protein abundance, we performed Western analysis of N2 and wdr-23 (tm1817) worms using an anti-SKN-1 monoclonal antibody (7). This antibody detects a 67.5-kDa protein that is sensitive to skn-1 RNAi (Fig. 7B). As shown in Fig. 7C, wdr-23 loss of function increased SKN-1 protein levels by 2.8-fold, demonstrating that WDR-23 regulates SKN-1 protein abundance.

WDR-23 functions independently of DAF-16. Like SKN-1, the FOXO transcription factor DAF-16 accumulates in the cell nuclei in response to stressors (29, 35), including juglone (Fig. 8A). In addition, recent studies have demonstrated that gst-4 expression is regulated in part by DAF-16 (55). These results suggest that WDR-23 may play a role in regulating DAF-16. To test this possibility, we fed DAF-16::GFP-expressing worms wdr-23 dsRNA bacteria. Figure 8A demonstrates that loss of wdr-23 function does not promote nuclear accumulation of DAF-16. To test this possibility, we fed DAF-16::GFP-expressing worms wdr-23 dsRNA bacteria. Figure 8A demonstrates that loss of wdr-23 function does not promote nuclear accumulation of DAF-16. In addition, Pgst-4::GFP expression in wdr-23 deletion mutants was not inhibited by DAF-16 RNAi (Fig. 8B), which is sufficient to eliminate DAF-16::GFP expression (Fig. 8C). Finally, RNAi of wdr-23 increased gst-4 and gst-30 mRNA levels in a daf-16 deletion mutant (mu86) (Fig. 8D), verifying that DAF-16 is not required for target gene activation. Taken together, the
data in Fig. 8 demonstrate that DAF-16 is not regulated by WDR-23 and indicate that loss of WDR-23 function induces gst-4 transcription specifically through SKN-1.

WDR-23 may function downstream from GSK-3, DAF-2, and SEK-1. Previous studies have identified protein kinase pathways that phosphorylate SKN-1 and alter its nuclear accumulation (2, 23, 55). However, it is unclear how SKN-1 phosphorylation contributes to nuclear accumulation and activation during stress. The data in Fig. 2 to 7 suggest that WDR-23 represses SKN-1 nuclear accumulation by recruiting it to the CUL-4/DDB-1 ubiquitin ligase complex and demonstrate that disruption of this complex is by itself sufficient to promote nuclear accumulation and activation. To determine if stressors activate gst-4 transcription via WDR-23 regulation, we exposed VP579 wdr-23 dsRNA-producing bacteria, paraquat, and juglone and measured Pgst-4::GFP fluorescence. As shown in Fig. 9A and B, none of these stressors, including juglone (Fig. 1), increased gst-4 transcription any more than wdr-23 loss of function alone. These results suggest that stressors activate gst-4 transcription through a mechanism that disrupts WDR-23 repression of SKN-1. In contrast, the effect of juglone on gst-4 expression was additive with RNAi of F28D1.1 (2.4-±0.05-fold induction; P<0.001), further supporting our conclusion that this WD40 protein functions in a pathway distinct from that of WDR-23.

Phosphorylation by GSK-3 and kinases downstream from the DAF-2 insulin-like pathway (AKT-1, AKT-2, and SGK-1) inhibit SKN-1 nuclear accumulation and activation (2, 55). To determine if these kinases repress SKN-1 in a pathway with WDR-23, we performed epistasis analysis using real-time PCR quantification of gst-4 mRNA, gsk-3 RNAi increased gst-4 mRNA by 3.3-fold in N2 worms but had no additive effect in wdr-23 loss-of-function animals (Fig. 9C). As shown by Tullet et al. (55), daf-2 loss of function increases gst-4 expression independently of daf-16 (Fig. 9D). However, daf-2 loss of function had no additive effect on gst-4 induction in wdr-23 RNAi worms (Fig. 9D). These data suggest that GSK-3 and insulin-like signaling may function together with WDR-23 to repress SKN-1 activity.
Nuclear accumulation and activation of SKN-1 require phosphorylation by the conserved p38 MAPK pathway (23). Oxidative stress activates the p38 MAPK PMK-1, which phosphorylates SKN-1 at residues required for its nuclear accumulation and activation (23). The MAPK kinase SEK-1 functions immediately upstream from PMK-1 and is required for oxidative-stress-induced activation of PMK-1 and SKN-1 (23). To determine if this pathway functions upstream from WDR-23, we quantified the effects of sek-1 loss of function on gst-4 and gst-30 induction in wdr-23 (RNAi) worms. As shown in Fig. 9E, sek-1 loss of function did not suppress wdr-23 (RNAi) induction of gst-4 or gst-30, suggesting that WDR-23 regulates SKN-1 downstream from the p38 MAPK pathway.

WDR-23 regulates stress resistance, longevity, and growth. Loss of skn-1 function reduces stress tolerance and life span (1, 2, 55), while overexpression of SKN-1 enhances stress tolerance and increases longevity (2, 55). Given that WDR-23 strongly represses SKN-1 activity (Fig. 2, 6, and 7), we predicted that loss of wdr-23 function would enhance stress tolerance and extend life span. As shown in Fig. 10A, wdr-23 RNAi increased the survival of worms exposed to peroxide and juglone by 1.2- and 2.6-fold (P < 0.05), respectively. Knockdown of wdr-23 by RNAi also significantly extended the median and mean life spans by 4.0 and 2.4 days, respectively (Fig. 10B). A similar extension of life span by wdr-23 knockdown has been observed in a recent RNAi screen for longevity genes (10). Given that WDR-23 functions normally to repress stress...
Our studies have defined a novel pathway that regulates the stress resistance and longevity factor SKN-1. The WD40 protein WDR-23 functions together with CUL-4, DDB-1, and the proteasome to repress SKN-1-dependent transcription of phase II detoxification genes (Fig. 2 and 4 to 6). WDR-23 physically interacts with SKN-1 (Fig. 6) and DDB-1 (Fig. 5), and loss of WDR-23 function causes accumulation of SKN-1 in intestinal nuclei (Fig. 6C), elevated SKN-1 protein levels (Fig. 7C), greatly increased expression of phase II detoxification genes (Fig. 2C), and increased stress resistance and longevity (Fig. 10).

A working model illustrating the proposed mechanism by which WDR-23 regulates SKN-1 is shown in Fig. 11. SKN-1 transcriptional activity is correlated with its accumulation in cell nuclei (1, 15, 23). Nuclear accumulation of SKN-1 occurs within minutes of oxidative stress and has led to the hypothesis that stress-induced translocation of SKN-1 from the cytoplasm to the nucleus is responsible for activation (1). Our data instead suggest that SKN-1 constitutively enters the nucleus. We propose that under nonstress conditions, nuclear accumulation of SKN-1 is prevented by WDR-23, which interacts with the CUL-4/DDB-1 complex and presumably targets the transcription factor for proteasomal degradation (Fig. 11A). Recent studies by Kahn et al. (27) demonstrated that the proteasome regulates SKN-1 nuclear accumulation. Our study confirms their results and defines a pathway that may target SKN-1 to the proteasome. Confirmation of our model will require demonstration of WDR-23-dependent ubiquitylation and degradation of SKN-1.

SKN-1 activity is regulated by phosphorylation through multiple protein kinase signaling pathways that respond to a diverse range of systemic and cellular stress, metabolic, and developmental signals (2, 23, 24, 31, 55). How can multiple signaling events be integrated to regulate SKN-1 activity? Our epistasis data raise the possibility that WDR-23 functions downstream from GSK-3, DAF-2, and p38 MAPK kinase cascades (Fig. 9). Phosphorylation of SKN-1 at multiple sites could alter the binding affinity between SKN-1 and WDR-23 and/or the ability of CUL-4/DDB-1 to ubiquitinylate the protein and target it for degradation. Multisite phosphorylation could thus fine-tune the SKN-1 half-life and transcriptional activity.

All previously characterized examples of CUL4-mediated ubiquitylation have been shown to occur on chromatin. CUL4/DDB1 is recruited to chromatin by chromatin-based signals that also regulate its activity (48). These findings, together with our data, suggest that WDR-23/CUL-4/DDB1 may interact with SKN-1 while it is regulating the transcription of target genes. This would provide an important mechanism for terminating SKN-1 activity at the site of gene transcription.

Our data (Fig. 9A) suggest that under stress conditions SKN-1 may activate gene transcription by escaping WDR-23/CUL-4/DDB1 repression (Fig. 11B). This could occur through phosphorylation of SKN-1 by PMK-1 (23) and/or by direct modification of WDR-23. Interestingly, many redox sensors function by forming adducts with electrophiles through reactive cysteines (22). C. elegans WDR-23 has 17 cysteine residues (Fig. 3B). Four of these cysteines are adjacent to basic amino
acids, which is a shared characteristic of reactive cysteines that is thought to increase sensitivity to electrophiles (20). Direct modification of WDR-23 by electrophiles could disrupt its interaction with SKN-1, rapidly increasing the stability and activity of the transcription factor under conditions that require robust expression of detoxification genes (Fig. 11B). Interestingly, the 5′ regulatory region of the \( wdr-23 \) gene contains five potential SKN-1 binding elements (6), and we have observed that \( wdr-23 \) mRNA levels are elevated 2.5-fold by juglone (unpublished observations). These observations suggest a simple autoregulation loop in which activation of SKN-1 promotes the expression of unmodified WDR-23 that could replace electrophile-inactivated protein and reduce and terminate nuclear SKN-1 activity as electrophiles are detoxified and excreted.

As shown in Fig. 10C, loss of WDR-23 activity causes a slow-growth phenotype (see also reference 52) that is partially suppressed by \( skn-1 \) RNAi. This suggests that tight regulation of SKN-1 transcriptional activity by WDR-23 is essential for normal growth and development.

CUL4/DDB1 ubiquitin ligases interact with DNA and histones to regulate DNA repair, DNA replication, and chromatin remodeling (19, 37). This occurs through degradation of the DNA replication-licensing factor CDT1 and through substrate adaptors that alter ubiquitylation and methylation of histones (48). Regulation of SKN-1 by CUL-4/DDB-1 provides a potential mechanism for coordinating cytoprotective gene expression with DNA repair, the cell cycle, and chromatin modifications. Coordination of SKN-1 target gene transcription with DNA repair would be advantageous, given that oxidative stress and xenobiotics can cause DNA damage.

**Does WDR23 regulate Nrf2?** Transcription of phase II detoxification genes in mammals is predominantly activated by the NF-E2-related factor (Nrf) basic-leucine zipper protein Nrf2 (34). Nrf2 protects mammals from oxidative stress and age-related diseases, such as cancer, neurodegeneration, and chronic inflammation (34, 39). In addition, aberrant Nrf2 activity has recently been implicated in the development of multidrug resistance in cancer cells (16, 53, 57). These observations have made pharmacological modulation of Nrf2 an important approach for treating several human diseases (58). Detailed molecular characterization of all Nrf2 regulatory pathways is thus essential for gaining a comprehensive understanding of its roles in disease and for developing new therapeutic strategies and targets.

It is interesting to speculate that human WDR23 may perform a role similar to that of \( C. \) elegans WDR-23. Nuclear accumulation and activity of Nrf2 in Drosophila and vertebrate cells are controlled by Keap1, which functions as an actin binding protein and as a substrate recognition subunit for the Cullin ubiquitin ligase CUL3 (34, 49). Keap1 functions with CUL3 ubiquitin ligases in the cytoplasm (34, 49) and may also transiently shuttle into the nucleus (46).

Studies with Nrf2 mutants that do not bind to Keap1 demonstrate that it is targeted for degradation by at least one Keap1-independent mechanism (43, 47). \( C. \) elegans SKN-1 and mammalian Nrf2 share regions of high homology, including one that is postulated to mediate Keap1-independent ubiquitylation in the nucleus (43). Humans have one WDR23 orthologue (Fig. 3), and it interacts with CUL4/DDB1 ubiquitin ligases (3). It will be critical to determine if WDR23 or other nuclear CUL4/DDB1 substrate adaptors regulate Nrf2 in mammals, which would allow temporal and spatial specialization of Nrf2 regulation.
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REFERENCES


