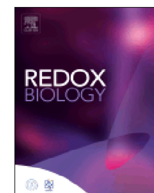
Redox Biology 21 (2019) 101111

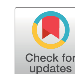
Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox



Arginine methylation of SKN-1 promotes oxidative stress resistance in *Caenorhabditis elegans*



Hongyuan Li[a,c,1], Liangping Su[a,d,1], Xin Su[a], Xin Liu[e], Dan Wang[a], Hongmei Li[a], Xueqing Ba[a], Yu Zhang[b], Jun Lu[b], Baiqu Huang[b,*], Xiaoxue Li[a,*]

[a] The Key Laboratory of Molecular Epigenetics of the Ministry of Education, Northeast Normal University, Changchun 130024, China
[b] The Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, China
[c] Laboratory of Chemical Biology, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China
[d] School of Basic Medical Sciences, Guilin Medical University, Guilin 541004, China
[e] College of Life Sciences, Engineering Research Center of the Chinese Ministry of Education for Bioreactor and Pharmaceutical Development, Jilin Agricultural University, Changchun 130118, China

ARTICLE INFO

Keywords:
SKN-1
PRMT-1
Arginine methylation
Oxidative stress

ABSTRACT

Caenorhabditis elegans NRF (NF-E2-related factor)/CNC (Cap'n'collar) transcription factor, Skinhead-1 (SKN-1), is conservatively critical for promoting phase II detoxification gene expressions in response to oxidative stress. SKN-1 activity is controlled by well-known phosphorylation and recently-reported *O*-GlcNAcylation. Whether other kinds of posttanslational modifications of SKN-1 occur and influence its function remains elusive. Here, we found arginines 484 and 516 (R484/R516) of SKN-1 were asymmetrically dimethylated by PRMT-1. Oxidative stress enhanced the binding of PRMT-1 to SKN-1. Consequently, asymmetrical dimethylation of arginines on SKN-1 was elevated. Loss of *prmt-1* or disruption of R484/R516 dimethylation decreased the enrichment of SKN-1 on the promoters of SKN-1-driven phase II detoxification genes, including gamma-glutamine cysteine synthetase *gcs-1*, glutathione S-transferases *gst-7* and *gst-4*, which resulted in reduced ability of worms to defense against oxidative stress. These findings have important implications for investigating the physiological and pathological functions of arginine methylation on conserved NRF/CNC transcription factors in human diseases related to oxidative stress response.

1. Introduction

Oxidative stress is a major factor involved in human diseases, such as diabetes, cancer, and neurodegenerative diseases [1–3]. From worms to humans, eukaryotic cells respond to oxidative stress by promoting the activity of NRF (NF-E2-related factor)/CNC (Cap'n'collar) transcription factor family [4,5]. This family contributes to activate expressions of phase II detoxification genes, which encode enzymes that synthesize glutathione, scavenge free radicals, and detoxify reactive products of phase I (P450) system, including UDP-glucuronosyltransferases, glutamine cysteine synthetase, and glutathione S-transferases [6,7].

As a NRF/CNC transcription factor, Skinhead-1 (SKN-1) orchestrates the conserved detoxification response in *Caenorhabditis elegans* (*C. elegans*) [7–9]. At the earliest embryonic stages, SKN-1 is present in nuclei of intestinal precursors, and initiates the development of feeding and digestive system (intestine) [7]. During larval and adult stages, SKN-1 accumulates in the intestinal nuclei and promotes expressions of phase II detoxification genes, such as gamma-glutamine cysteine synthetase *gcs-1*, glutathione S-transferases *gst-7* and *gst-4*, which play key roles in increasing oxidative stress resistance and extending lifespan [7,8,10,11].

Numerous evidence shows that transcriptional activities of NRF/CNC family are evolutionarily controlled by a few of posttranslational modifications, including phosphorylation, ubiquitination and acetylation [12–17]. During antioxidative response, SKN-1 activity is well-

Abbreviations: NRF, NF-E2-related factor; CNC, Cap'n'collar; SKN-1, Skinhead-1; PRMT-1, protein arginine methyltransferase 1; ADMA, asymmetrically dimethylated arginine; SDMA, symmetrically dimethylated arginine; *gcs-1*, glutamine cysteine synthetase 1; *gst-4*, glutathione S-transferase 4; *gst-7*, glutathione S-transferase 7; OGT-1, *O*-linked N-acetylglucosamine transferase 1; tBHP, t-butyl hydrogen peroxide; IPTG, isopropyl-β-D-thiogalactopyranoside; NGM, nematode growth media; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; qPCR, quantitative PCR; GSK-3, glycogen synthase kinase-3; IIS, insulin/IGF-1

* Corresponding authors.
E-mail addresses: huangbq705@nenu.edu.cn (B. Huang), lixx956@nenu.edu.cn (X. Li).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.redox.2019.101111
Received 30 November 2018; Received in revised form 9 January 2019; Accepted 13 January 2019
Available online 15 January 2019

2213-2317/ © 2019 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).

known modulated by its phosphorylation in a few of signaling pathway [10,11,18]. Recently, we report that *O*-GlcNAcylation catalyzed by *O*-GlcNActransferase OGT-1 enhances SKN-1 accumulation in intestinal nuclei to defense against oxidative stress [19]. Whether other kinds of posttanslational modifications influence SKN-1 transcriptional activity remains poorly understood.

Arginine methylation is a widespread protein posttranslational modification, which has been well revealed in various cellular processes, including signal transduction, transcriptional regulation and DNA repair pathway [20–24]. This modification is catalyzed by a family of protein arginine methyltransferases (PRMTs) that add one or two methyl groups to the guanidine nitrogen atoms of arginine [24–26]. Protein arginine methyltransferase 1 (PRMT-1) is a type I PRMTs that produce asymmetrically dimethylated arginine (ADMA) on the target proteins [20]. Recently, we find that PRMT-1-catalyzed arginine methylation of human NRF/CNC factors, NRF2 (NF-E2-related factor 2), promotes antioxidative response in cancer cells [27]. The physiological significance of arginine methylation on NRF/CNC family remains elusive because of the lethal phenotypes of null alleles of mouse *prmt-1* or *Drosophila* homolog *dart1* [28,29]. In the light of normal embryonic and larval development of nematode *prmt-1* mutants [30], *C. elegans* become a good model to explore the physiological function of arginine methylation on NRF/CNC family in organisms.

Here, we found that in response to oxidative stress induced by t-butyl hydrogen peroxide (tBHP), the binding of PRMT-1 to SKN-1 was enhanced, resulting in an increased asymmetrical dimethylation of arginines on SKN-1. Mass spectrometry showed that arginines 484 and 516 (R484/R516) of SKN-1 were predominantly dimethylated by PRMT-1. Methylation of R484/R516 elevated the recruitment of SKN-1 on the promoters of phase II detoxification genes, and increased oxidative stress resistance in worms. These findings imply that asymmetrical dimethylation of arginines on NRF/CNC family plays critical function during antioxidative response, which is conserved from worms to human cancer cells.

2. Materials and methods

2.1. Strains

The following strains were used in this work: N2 Bristol (wild-type), *prmt-1(ok2710)*; LD1171 (*ldls3*, *Pgcs-1*::GFP), LD1(*ldls7*, SKN-1B/C::GFP) were provided by the Caenorhabditis Genetic Center (CGC), *prmt-1(ok2710)* were crossed to LD1(*ldls7*) to generate *prmt-1(ok2710);ldls7* strains. The strain of *prmt-1(ok2710)* from CGC was backcrossed at least three times before used. The transgenic worms: N2*[rol-6]*, N2*[SKN-1B/C::GFP;rol-6]*, N2*[SKN-1B/C R484K/R516K::GFP;rol-6]*, *skn-1(zu67)[rol-6]*, *skn-1(zu67)[SKN-1B/C::GFP;rol-6]*, *skn-1(zu67)[SKN-1B/C R484K/R516K::GFP;rol-6]* were made by following transgene procedures.

2.2. Transgenes

The plasmid *SKN-1B/C::GFP* was a generous gift from Prof. T. Keith Blackwell (Research Division, Joslin Diabetes Center). The double-sites mutations of *SKN-1B/C R484K/R516K*::*GFP* were created by using the protocol of QuikChange (Stratagene). 10 ng/μl of each plasmid was injected into wild-type (N2) and *skn-1(zu67)* mutant worms, along with the *rol-6* marker (pRF4) at 50 ng/μl.

2.3. RNAi

RNAi clones were grown in LB with 12.5 μg/ml tetracycline and 100 μg/ml ampicillin. On the next day, cultures were diluted and grown to an OD600 of 1, and induced with 4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). This culture was used to seed plates containing ampicillin, and additional IPTG, and then left to dry for 1–2 days at room temperature.

2.4. Lifespan analysis

Prior to experiments, all animals were maintained at the permissive temperature and grown for at least two generations in the presence of food to assure health. Lifespan analyses were conducted at 20 °C. Synchronized L1 worms were fed with OP50, grown to young adult, and then 30 worms were transferred to a new plate. Animals were tapped every day and scored as dead when they did not respond to the platinum wire pick. All of the lifespan assays were repeated at least three times. Survival plots, p values (Log-Rank), and proportional hazards were determined by using GraphPad Prism 5 software.

2.5. Oxidative stress resistance assay

To assess oxidative stress resistance in solid culture medium, day 1 post adults were transferred to plates containing 9.125 mM t-butyl hydrogen peroxide (tBHP) (Sigma) in NGM (nematode growth media). These plates had been seeded with OP50. Animals were incubated on these plates at 20 °C and periodically scored for survival. To assess this stress resistance in liquid culture medium, young adults were transferred to 1 ml M9 buffer that contained 5 mM tBHP in the tube for 90 min.

2.6. Fluorescence microscope assays

The young adults of *Pgcs-1*::GFP worms were fixed by 2 mM levamisole and then taken photographs under fluorescence microscope with identical camera gain and exposure settings. The patterns of GFP expression driven by *gcs-1* promoter were assessed as “high”, “medium” and “low” according to the protocol in the previous study [10]. “High” indicates that GFP was present at high levels anteriorly and was detectable in the intestine. “Medium” refers to animals in which GFP was present at high levels anteriorly and possibly posteriorly but was not detected in between. “Low” refers to animals in which GFP was barely detected. The percentage of each *Pgcs-1*::GFP patterns was analyzed by a chi2 test, and the differences were considered as significance at $p < 0.05$.

2.7. RNA isolation and quantitative PCR (qPCR)

To prepare worm sample, animals were picked and placed to clean plates to minimize contamination. Then approximately 200 animals suspended in 50 μl M9 buffer. Total RNAs from worms were prepared by using Trizol reagent kit (TaKaRa) according to the manufacturer's instructions. The cDNA was generated with oligo(dT) primers (Promega) by using the Reverse Transcription System (Promega). The Quantitative Real Time PCR was carried out using the SYBR Green Real time PCR Master Mix (TOYOBO) on a Light Cycler 480 Real-Time PCR system (Roche). The mRNA expression levels of *gcs-1*, *gst-4* and *gst-7* were normalized by the geometric mean of the expression of *cdc-42*, *pmp-3* and *Y45F10D.4* [31]. The primers sequences for PCR were shown in Supplementary Table S1.

2.8. Plasmids, reagents and antibodies

The cDNAs for wild-type SKN-1, truncated SKN-1 peptides, and PRMT-1 of *C. elegans* were amplified by PCR and cloned into pGEX-6P-1. All of the point mutations of SKN-1 were generated by site-directed mutagenesis according to the QuikChange (Stratagene) protocol. The antibodies of anti-GFP, anti-GST (Tianjin Sungene Biotech), anti-β-actin (Sigma), anti-ASYM24 (Millipore) and anti-PRMT1 (Upstate) were purchased from the commercial channels.

2.9. In vitro pull-down assays

The proteins of GST and GST-PRMT-1 were expressed in BL21(DE3) bacteria and purified with glutathione Sepharose 4B beads (for GST fusion proteins, GE Healthcare), respectively. GST-fusion protein was incubated with the whole worm lysates of LD1(*ldls7*) and glutathione Sepharose 4B beads. After extensive washes, bound proteins were analyzed by western blotting with an anti-GFP antibody. Twenty percent of proteins used for the pull down reaction were shown as input.

2.10. Co-Immunoprecipitation and western blots

To prepare *C. elegans* proteins, synchronized young adult worms were grown on 9.5 cm plates at 20 °C and washed off from the plates with M9 buffer. The worms were lysed by sonication in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, phosphatase inhibitors, and protease inhibitors) and then immunoprecipitated with anti-GFP antibody, followed by western blotting. For immunoprecipitation, total protein lysates were incubated overnight with corresponding antibodies with gentle shacking at 4 °C followed by addition of 40 μl of Pure Proteome protein A/G Mix Magnetic Beads (Millipore) for another 3 h. The beads were resuspended in 60 μl of 2 ×loading buffer and boiled for 10 min. Then, the supernatant was subjected to SDS-PAGE, transferred to polyvinylidene fluoride membrane (Millipore) and visualized by using appropriate primary antibodies coupled with HRP-conjugated secondary antibodies by ECL reagent (GE Healthcare).

2.11. In vitro methylation assay and mass spectrometry analysis

Each of attenuated GST-SKN-1 proteins and GST-PRMT1 and were expressed in bacteria (BL21) induced by isopropyl-β-D-thiogalactoside and then purified as described previously, respectively. The recombinant full-length or fragmental GST-SKN-1 (5 μg), or core histone (1 μg) were incubated with 3 μg of GST-PRMT1 in the presence of ^{3}H-S-adenosyl-methionine (15 Ci/mMol, Perkin-Elmer) at 30 °C for 1 h. The reaction products were analyzed by autoradiography and CBB staining. For Mass Spectrometry, the Coomassie blue staining band of GST-SKN-1 incubating with GST-PRMT1 plus S-adenosyl-methionine (Sigma) was excised for LC-MS/MS analysis performed in the Academy of Military Medical Sciences, Beijing.

2.12. Electrophoretic mobility shift assay (EMSA)

GST-PRMT-1(3 μg) and GST-SKN-1(321–624aa) (3 μg) were purified with glutathione Sepharose 4B beads (for GST fusion proteins, GE Healthcare), respectively. The sequence of the *gcs-1* gene probe was designed according to published literature (5′-AAATATCACTTTATCA TCATGAGAT-3′) [8]. The labeling of probe and electrophoretic mobility shift assays were carried out according to the manufacturer's instructions of Biotin 3′ End DNA Labeling Kit and LightShift Chemiluminescent EMSA Kit (Thermo Scientific).

2.13. Chromatin immunoprecipitation (ChIP)-quantitative PCR (qPCR)

Synchronized young adults worms were subjected to five freeze/thaw cycles in liquid nitrogen, and cross-linked with 2% formaldehyde for 20 min as described [32]. After cross-linking was stopped with glycine, samples were washed with PBS and lysed for 10 min with buffer containing 50 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS and protease inhibitor cocktail (Roche). Lysates were sonicated by using a Scientz-D (Amplitude 60; 25 cycles; 15 s 'on', 45 s 'off'; 4 °C). Cleared lysates were immunoprecipitated using anti-IgG (Santa sc-2027), anti-GFP (Abcam ab1218), and magnetic beads (Protein A/G dynabeads, Invitrogen). After proteinase K digestion and reverse cross-linking, DNA was purified by phenol/chloroform extraction and used for qPCR. The primers used in ChIP-qPCR were shown in Supplementary Table S1. p values were analyzed by GraphPad Prism 5 software and determined by Student's *t*-test.

2.14. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Data were presented as mean ± SEM. For RT-qPCR assays, p values were determined by Student's *t*-test. For oxidative stress resistance and lifespan assays, p values were determined by log-rank test. For the pattern of *Pgcs-1*::GFP expression analysis, a chi2 test was used, and the differences were considered as significance at $p < 0.05$.

3. Results

3.1. PRMT-1 promotes activity of SKN-1 in driving phase II detoxification gene expressions during antioxidative response

To explore the influence of PRMT-1 on SKN-1 activity, we detected the changes of SKN-1 target phase II detoxification gene expressions in *prmt-1* mutant worms during antioxidative response. t-butyl hydrogen peroxide (tBHP) was utilized as an oxidative stress inducer according to previous publications [8,10,18]. Results showed that the mRNA levels of phase II detoxification genes controlled by SKN-1, including gamma-glutamine cysteine synthetase *gcs-1*, glutathione S-transferases *gst-4* and *gst-7*, were increased by tBHP treatment in wild-type (N2) worms; and these tBHP-induced increases were abolished by loss of *prmt-1* (Fig. 1**A**).

Using *Pgcs-1::GFP* transgenic worms, in which the green fluorescent protein (GFP) expression is activated by binding of SKN-1 on *gcs-1* promoter, we found tBHP treatment elevated the transcriptional activity of SKN-1 in driving GFP expression. This tBHP-induced increase of SKN-1 activity was attenuated by *prmt-1* RNAi (Fig. 1**B-C**). These indicate that PRMT-1 plays a key role in promoting SKN-1 activity of phase II detoxification gene transcription during antioxidative response.

3.2. Oxidative stress increases the binding of PRMT-1 to SKN-1, and elevates asymmetrical dimethylation of arginines on SKN-1

To explore whether PRMT-1 directly interacts with SKN-1, we operated GST pull-down by utilizing the whole-worm extracts of wild-type (N2) worms expressing SKN-1(B/C)::GFP, and found SKN-1 bound to GST-PRMT-1, but not GST *in vitro* (Fig. 2**A**). SKN-1(B/C)::GFP proteins were then immunoprecipitated by GFP antibody from N2[SKN-1(B/C)::GFP] worms, followed by immunoblotting with PRMT-1 antibody and ASYM antibody that specifically recognizes asymmetrically dimethylated arginines. Results showed that SKN-1 interacted with PRMT-1 *in vivo*, and asymmetrical dimethylation of arginines was observed on SKN-1 proteins expressed in N2 worms (Fig. 2**B**).

To investigate whether asymmetrical dimethylation of arginines on SKN-1 is changed during antioxidative response, we treated N2[SKN-1(B/C)::GFP] worms with tBHP, followed by co-immunoprecipitation experiments. Results showed that tBHP treatment enhanced the binding of PRMT-1 to SKN-1, and increased asymmetrical dimethylation of arginines on SKN-1 (Fig. 2**C**). These imply that arginine dimethylation of SKN-1 catalyzed by PRMT-1 acts as a physiologically antioxidative respond in *C. elegans*.

3.3. Arginines 484 and 516 of SKN-1 are primary sites methylated by PRMT-1

To identify the specific arginine sites of SKN-1 that are methylated by PRMT-1, we performed ^{3}H-labeling methylation assay by incubation of GST-PRMT-1 with four truncated GST-SKN-1 peptides *in vitro*,

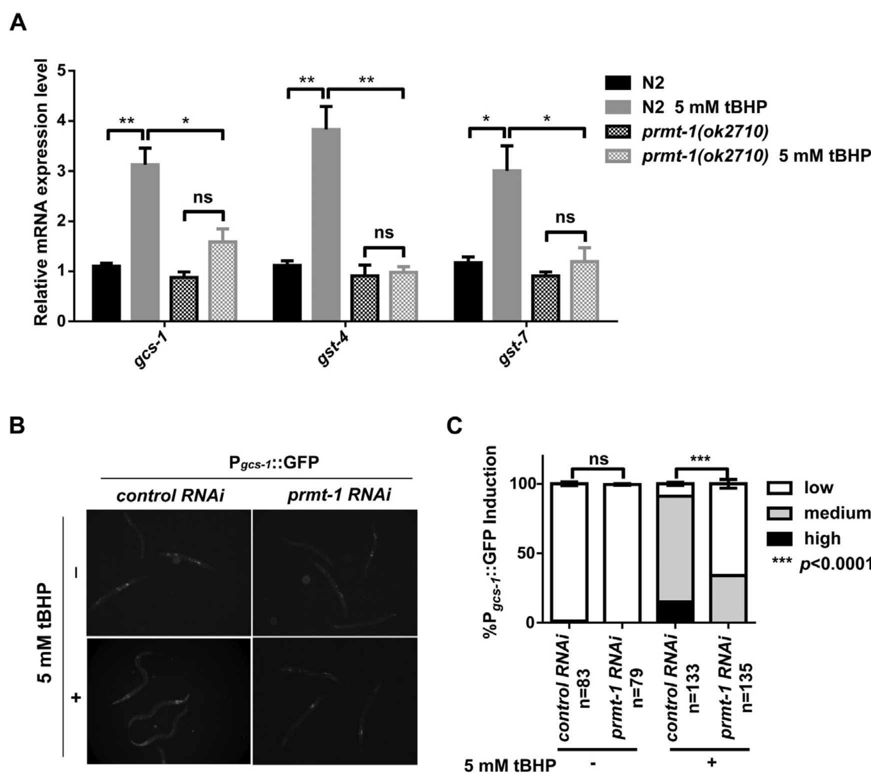


Fig. 1. PRMT-1 promotes SKN-1 activity of driving phase II detoxification gene expressions in response to tBHP-induced oxidative stress. (**A**) Loss of *prmt-1* attenuated the increase of SKN-1-driven phase II detoxification gene expressions induced by tBHP. tBHP was utilized as oxidative stress inducer according to previous publication [8,10,18]. Wild-type (N_2) and *prmt-1* mutants were treated with 5 mM tBHP for 90 min, followed by RT-qPCR analysis, which was normalized using the geometric mean of the expression of *cdc-42*, *pmp-3* and *Y45F10D.4*. Error bars represented the SEM of three independent replicates. ns, no significant; *, p < 0.05; **, p < 0.01. (**B-C**) *prmt-1* RNAi attenuated the tBHP-induced increase of SKN-1 activity in stimulating *gcs-1* promoter. The transgenic worms P_{gcs-1}::GFP, in which GFP protein expression is driven by *gcs-1* promoter, were treated upon control RNAi or *prmt-1* RNAi, followed by 5 mM tBHP treatment for 90 min. control RNAi, empty vector. The representative images of P_{gcs-1}::GFP expressions were shown in **B**. The percentages of P_{gcs-1}::GFP expression was assessed by "low", "medium" and "high" according to the previous studies [10], and analyzed in **C**. n, the number of worms for quantitative analysis. ***, p < 0.001.

respectively. Followed by autoradiography, we found GST-PRMT-1 methylated peptides of GST-SKN-1(1–90aa) and GST-SKN-1(321–623aa), but not GST-SKN-1(91–260aa) and GST-SKN-1(261–460aa) (Fig. 3**A**). The methylated 1–90aa and 321–623aa peptides obtained from *in vitro* methylation reactions were then analyzed by mass spectrometry. Results showed that arginines 484 and 516 (R484 and R516) of SKN-1 were methylated by PRMT-1 (Fig. 3**B-C**). We mutated arginines 484 and 516 into lysines to generate R484K or R516K single-site mutation, and R484K/R516K double-sites mutation (Fig. 3**D**). *In vitro* methylation assays showed that all these mutations abolished the asymmetrical dimethylation of arginines on SKN-1 (Fig. 3**E**, Fig. S1). These data illuminate that PRMT-1 predominantly

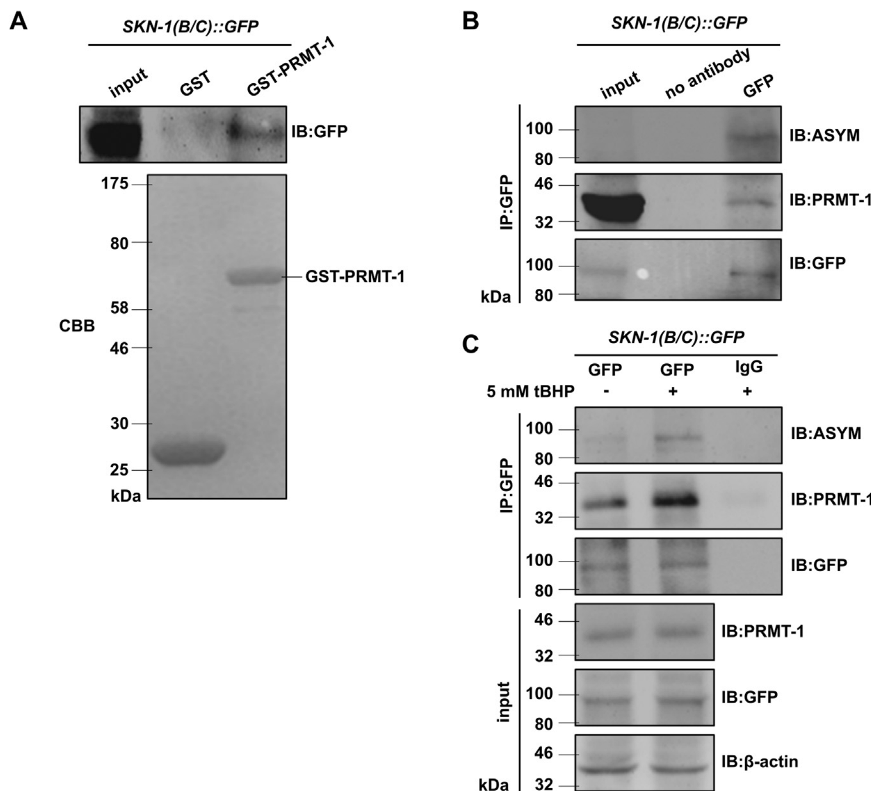


Fig. 2. Oxidative stress enhances binding of PRMT-1 to SKN-1, and elevates asymmetrical dimethylation of arginines on SKN-1. (A) GST-PRMT-1 bound to SKN-1 proteins expressed in wild-type worms. GST pull-down assay was performed by immunoprecipitation with GST-PRMT-1 protein from the whole extracts of N2[SKN-1B/C::GFP] worms, followed by immunoblotting with anti-GFP antibody. The arrow on the right indicated the band of GST-PRMT-1 purifed from *E. coli* BL21. GST protein was as the negative control. Coomassie brilliant blue (CBB) staining (lower) was used as a loading control. (B) SKN-1 interacted with PRMT-1 and was asymmetrically dimethylated on arginines *in vivo*. co-IP was operated by utilizing the whole extracts of N2[SKN-1B/C::GFP] worms with anti-GFP antibody, followed by immunoblotting with anti-PRMT-1 and anti-ASYM antibody. No antibody was used as the negative control. (C) Oxidative stress increased the binding of PRMT-1 to SKN-1, and elevated asymmetrical dimethylation of arginines on SKN-1. N2[SKN-1B/C::GFP] worms were treated with (or without) 5 mM tBHP, followed by co-IP as the legend to B. β-actin and GFP were utilized as internal references. IgG was used as the negative control.

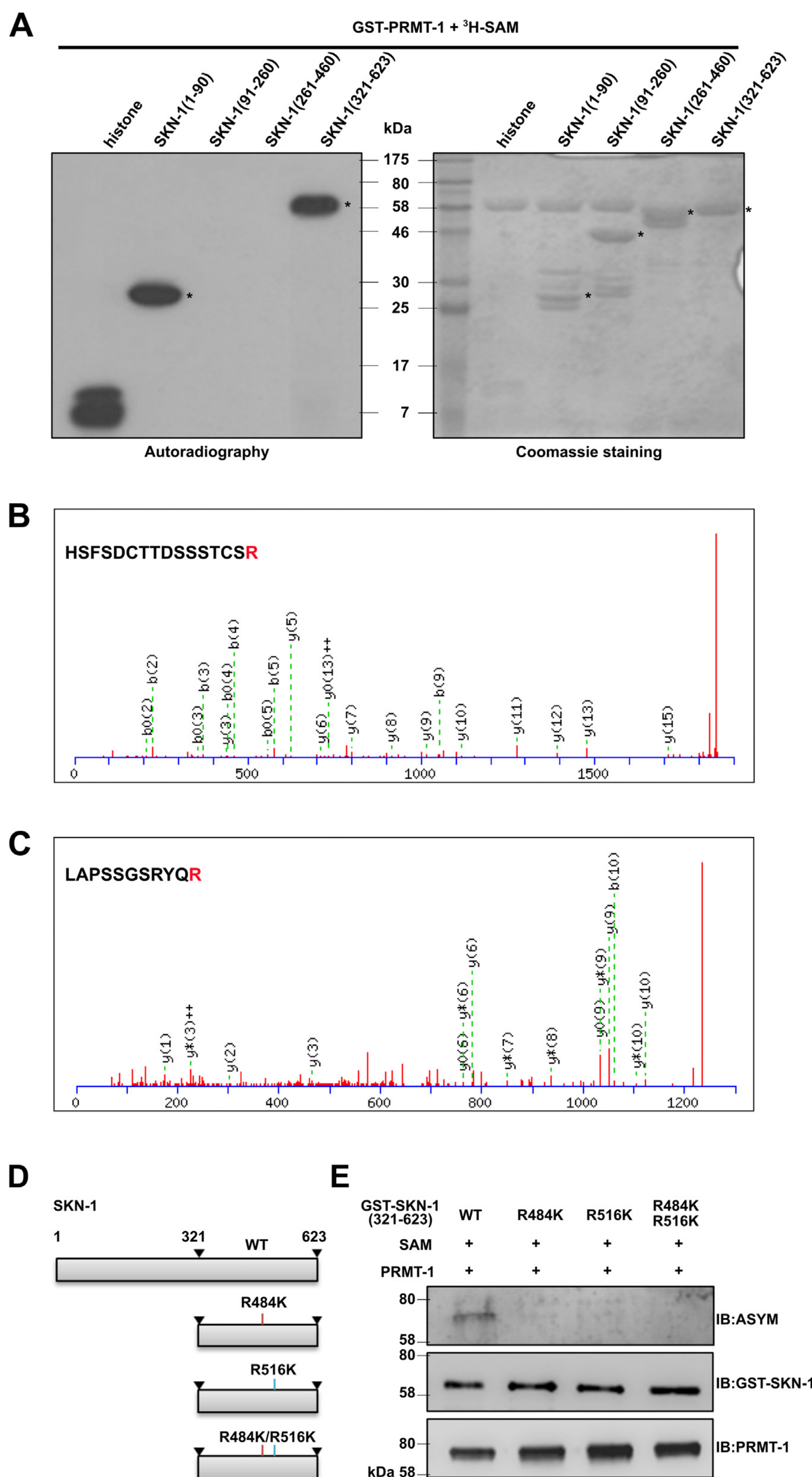


Fig. 3. R484 and R516 of SKN-1 are predominantly methylated by PRMT-1. (A) The truncated peptides of SKN-1(1-90aa) and SKN-1(321-623aa) were directly methylated by PRMT-1 *in vitro*. Methylation assays were performed by incubating GST-PRMT1 with four truncated peptides of GST-SKN-1 in the presence of ^{3}H-labeled S-adenosyl-methionine (SAM). Core histones were used as the positive control. The ^{3}H-labeled methylations of SKN-1 peptides were detected by autoradiography (left asterisks). Total amounts of GST-SKN-1 truncated peptides (right asterisks) were stained by CBB as loading control. (B-C) The arginine methylation sites of SKN-1 were analyzed by mass spectrometry. The residues of R484 and R516 (in red) were identified from two fragments of SKN-1 peptides. (D) R484 and R516 were the major methylation sites catalyzed by PRMT-1. R484 and R516 of SKN-1(321-623aa) peptides were single- or double- mutated into lysines (R484K, R516K, or R484K/R516K), followed by *in vitro* methylation reaction with GST-PRMT1 and unlabeled SAM. The asymmetrical dimethylation level of arginines on SKN-1 was detected by immunoblotting with anti-ASYM antibody, and quantified by densitometry analysis in Fig. S1. WT mean wild-type GST-SKN-1(321-623aa) peptides.

methylates R484 and R516 of SKN-1.

3.4. R484/R516 dimethylation enhances the enrichment of SKN-1 on phase II detoxification gene promoters

During antioxidative response, SKN-1 accumulates in intestinal nuclei and binds to the promoters of phase II detoxification genes to activate these gene expressions [8]. Disruption of R484/R516 methylation via R484K/R516K double-sites mutation had no effect on SKN-1 expression (Fig. S2) and its intestinal nuclear accumulation (Fig. S3). However, Chromatin immunoprecipitation (ChIP)-qPCR results showed that loss of *prmt-1* decreased the enrichment of SKN-1 on the promoters of SKN-1 target genes *gcs-1, gst-4* and *gst-7* (Fig. 4**A**), suggesting PRMT-1 enhances the binding of SKN-1 to these promoters.

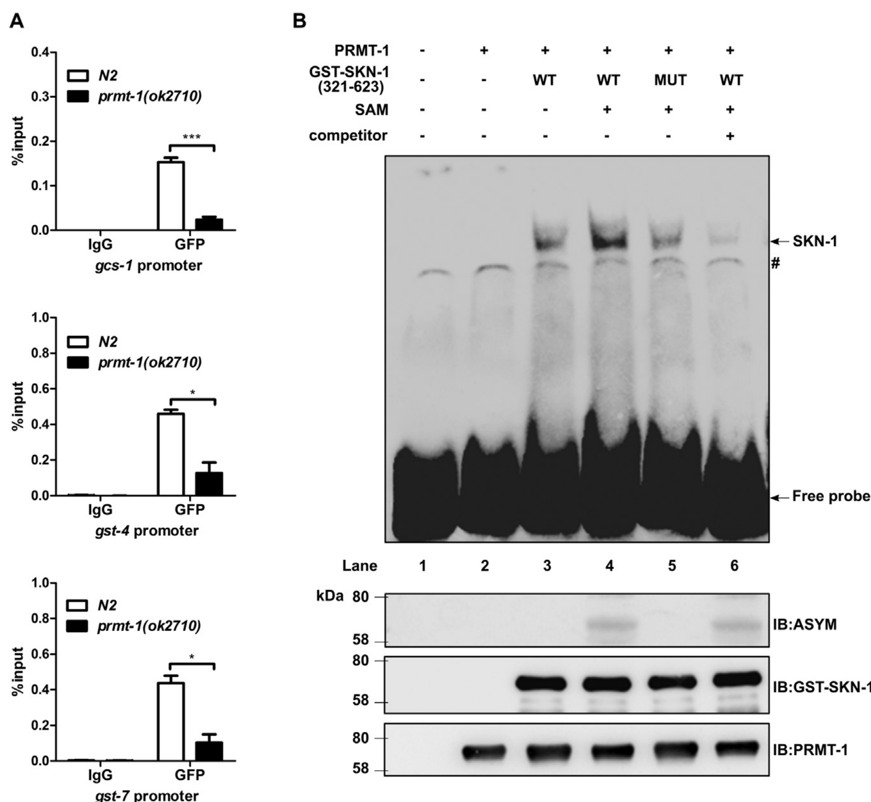


Fig. 4. R484/R516 dimethylation increases the binding of SKN-1 on phase II detoxification gene promoters. (A) Loss of *prmt-1* decreased the enrichment of SKN-1 on the promoters of phase II detoxification genes. ChIP-qPCR were performed with GFP antibody using N2 and *prmt-1(ok2710)* worms expressing SKN-1B/C::GFP. The levels of SKN-1B/C::GFP binding to the promoters of *gcs-1, gst-4* and *gst-7* were presented as percentage of the qPCR signal in total input DNA. IgG was used as the negative control. Error bars represent the SEM of three independent replicates. *, $p < 0.05$; ***, $p < 0.001$. (B) Disruption of R484/R516 dimethylation reduced the binding of SKN-1 on *gcs-1* promoter. Methylation assays were performed by incubating GST-PRMT1 with WT (wild-type) or MUT (R484K/R516K double-sites mutant) GST-SKN-1 (321-623aa) peptides *in vitro*, followed by EMSA assays (Upper panel) with biotin-labeled probe of *gcs-1* promoter. The upper arrow indicated the position of specific retarded band. The lower arrow indicated the free probe. # mean the unspecific band. Competition experiments (Lane 6) were performed by utilizing a 150-fold excess of unlabeled probe of *gcs-1* promoter. The asymmetrical arginines dimethylation levels of SKN-1 in each EMSA reaction were detected by western blots analysis with anti-ASYM antibody (Lower panel).

Utilizing the biotinylated DNA probes of *gcs-1* promoter and purified GST-SKN-1(321–623aa) peptides that has been previously methylated *in vitro* by GST-PRMT-1, we operated electrophoretic mobility shift (EMAS) assays. The PRMT-1-catalyzed arginine dimethylation of SKN-1 was detected by immunobloting the *in vitro* methylation reactions with anti-ASYM antibody **(**Fig. 4**B lower panel)**. Results showed that SKN-1 specifically bound to *gcs-1* promoter, and their binding was decreased by the unlabeled competition probes **(**Fig. 4**B upper panel, lane 3 vs lane 6)**. PRMT-1-mediated arginine methylation enhanced the binding of SKN-1 to *gcs-1* promoter **(**Fig. 4**B upper panel, lane 3 vs lane 4)**, and this increase was attenuated by disruption of R484/R516 methylation via R484K/R516K double-sites mutation **(**Fig. 4**B upper panel, lane 4 vs lane 5)**. These findings indicate that R484/R516 dimethylation catalyzed by PRMT-1 promotes the recruitments of SKN-1 to the promoters of phase II detoxification genes.

3.5. R484/R516 dimethylation increases the activities of SKN-1 in oxidataive stress resistance and lifespan extension

In order to explore whether R484/R516 dimethylation of SKN-1 influences the ability of oxidative stress resistance in worms, R484K/R516K double-sites mutations were generated in SKN-1(B/C)::GFP worms on N2 and *skn-1(zu67)* mutant background, respectively, followed by oxidative stress resistance assays. Results showed that wild-type SKN-1B/C::GFP overexpression increased the viability of N2 worms with tBHP treatment, and this increase was attenuated by disruption of R484/R516 methylation on SKN-1 via R484K/R516K double-sites mutations (Fig. 5**A,** Table S2). The capabilities of *skn-1(zu67)* mutants to defense against oxidative stress were restored by expression of wild-type SKN-1B/C::GFP, but not by R484K/R516K mutated SKN-1B/C::GFP (Fig. 5**B,** Table S3).

As increasing oxidative stress resistance is related to longevity [33–35], the lifespan assays were then detected in these transgenic worms described above. We found overexpression of wild-type SKN-1B/C::GFP extended the lifespan of N2, and this extension was abrogated by R484K/R516K mutations (Fig. 5**C,** Table S2). The shortened lifespan of *skn-1(zu67)* mutants was rescued by wild-type, but not R484K/R516K mutated SKN-1B/C::GFP (Fig. 5**D,** Table S3). These data demonstrate that PRMT-1-mediated R484/R516 dimethylation increases the activities of SKN-1 in oxidative stress resistance and extension of lifespan.

4. Discussion

Accumulating evidence shows that the activities of NRF/CNC transcription factor families are conservatively modulated by a few of posttranslational modifications during antioxidative response [12–17]. Although our group has recently reported that arginine methylation of human NRF/CNC transcription factor NRF2 increases the antioxidative activity of cancer cells [27]. Whether this modification on NRF/CNC families has evolutionary and physiological functions in organisms is poorly understood. In present study, we discovered a posttranslational modification, PRMT-1-mediated asymmetrical arginine dimethylation, of *C. elegans* NRF/CNC family member SKN-1, and found this modification is critical for promoting SKN-1 activity to respond oxidative stress. As the mouse with null alleles of *prmt-1* is lethal [29], these findings lay the foundation to further explore the roles of PRMT-1-catalyzed asymmetrical arginine methylation on NRF/CNC family in human diseases related to oxidative stress.

C. elegans PRMT-1 has been reported to maintain a normal lifespan by arginine methylation on FOXO forkhead transcription factor DAF-16 [30]. PRMT-1-mediated asymmetrical dimethylation of arginines increases DAF-16 translocation into nuclei by blocking DAF-16 phosphorylation via downstream serine/threonine kinases in Insulin/IGF-1 (IIS) pathway [30]. It has been proven that the intestinal nuclear accumulation of SKN-1 is inhibited by phosphorylation in IIS and glycogen synthase kinase-3 (GSK-3) pathway [10,11,18]. We recently report that GSK-3-mediated SKN-1 phosphorylation is repressed by *O*-GlcNAcylation of SKN-1, resulting in increasing accumulation of SKN-1 in intestinal nuclei [19]. Here, we observed that disruption of R484/

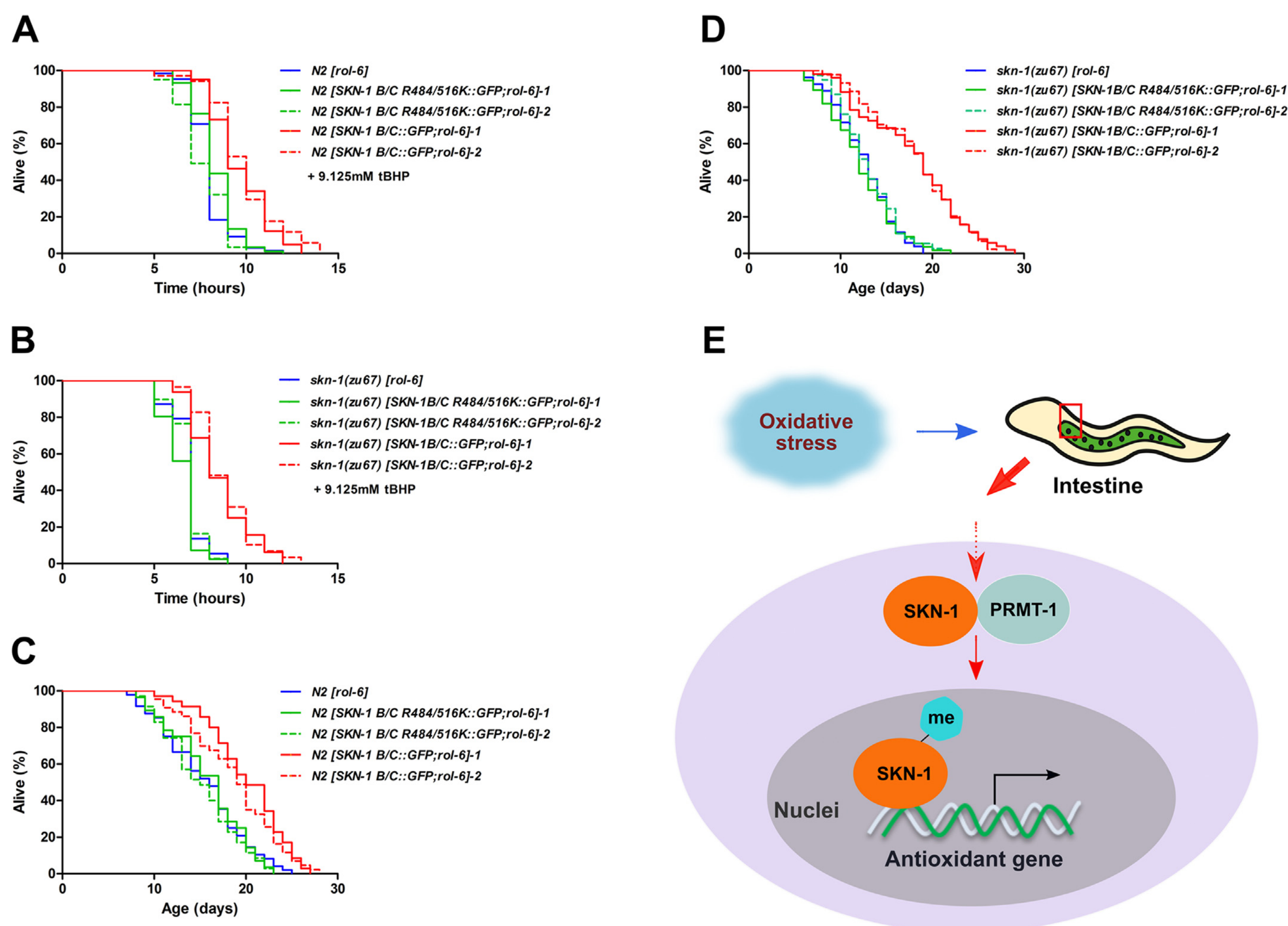


Fig. 5. R484/R516 dimethylation enhances the functions of SKN-1 in oxidataive stress resistance and longevity. (A and C) Disruption of R484/R516 dimethylation attenuated the increase of oxidataive stress resistance and extension of lifespan induced by SKN-1 overexpression. (B and D) Expression of R484K/R516K double-mutant SKN-1 failed to rescue the decreased oxidataive stress resistance and shortened lifespan of *skn-1(zu67)* mutant worms. 1# and 2# were two independent extrachromosomal arrays created by injecting plasmids SKN-1B/C::GFP and SKN-1B/C R484K/R516K::GFP with *rol-6* marker (pRF4) into wild-type (N2) and *skn-1(zu67)* mutants, respectively. The representative examples of oxidative stress resistance assays were shown in A and B. The representative examples of lifespan assays were shown in C and D. The statistical analysis based on three repeats was supplemented in Table S2 and Table S3. (E) The schematic model illustrating that arginine dimethylation by PRMT-1 promotes SKN-1 transcriptional activity during antioxidative response.

R516 methylation catalyzed by PRMT-1 did not change SKN-1 translocation into intestinal nuclei, but decreased the binding of SKN-1 to its target phase II detoxification gene promoters. These results imply that the role of R484/R516 methylation in promoting SKN-1 transcriptional activity is independent on SKN-1 phosphorylation. Therefore, SKN-1 phosphorylation level of worms lacking of R484/R516 methylation is worth further measuring.

Recent evidence has shown that PRMT-1 is responsible for the formation of asymmetric arginine dimethylation on mitochondrial proteins, including HSP-60, ATP-2, and Y38F1A.6 [36]. Moreover, a study, which determined the sub-cellular localization of about 227 body wall muscle proteins in *C. elegans*, revealed that PRMT-1 is localized in endoplasmic reticulum (ER) [37]. Here, our results showed that without tBHP treatment, PRMT-1 interacted with SKN-1 immunoprecipitated from N2[SKN-1(B/C)::GFP] transgenic worms. Since SKN-1 is retained in the intestinal cytoplasm without oxidative stress [8], these data suggested PRMT-1 bound to SKN-1 in the cytoplasm. It is well-known that cytoplasmic phosphorylation by both serine/threonine kinases AKT-1/2 and mitogen-activated protein kinase (MAPK) inhibits or promotes SKN-1 translocation into intestinal nuclei [11,18]. However, we found SKN-1 nuclear translocation was not altered by arginine methylation by PRMT-1; thus, the possibility that PRMT-1-mediated SKN-1 methylation also occurs in the nucleus could not be excluded.

Like the reported position of PRMT-1-mediated methylation site on human NRF2 (arginine 437) [27], the methylated residues R484 and R516 are close to the DNA binding domain of SKN-1 (Fig. S4). Here, we found that R484/516 dimethylation increased the binding of SKN-1 on its target phase II detoxification gene promoters. Whether this increase is controlled by promotion of SKN-1 recognizing target DNA or stabilization of DNA-SKN-1 complex is worth exploring in depth. Previous evidence shows that R597 and R609 are two major sites of SKN-1 to recognize DNA sequence [38]. R609 and its surrounded arginines (R593, R596, R597, R598, R611 and R612) prefer to stabilize the DNA-SKN-1 complex [38]. Disruption of R609 and its surrounded arginines will benefit the investigation of mechanism by which PRMT-1-catalyzed R484/516 dimethylation enhances the DNA binding activity of SKN-1.

In addition to asymmetrical arginine dimethylation, symmetrical arginine dimethylation are also reported to increase the DNA binding activities of transcription factors [39]. The production of symmetrically dimethylated arginine (SDMA) is catalyzed by type II PRMTs, including PRMT-5, PRMT-7, and FBXO11 in mammals [20]. The study in transcriptional factor E2F-1 shows that two methylation marks ADMA and SDMA generate opposing cell fate decision by targeting E2F-1 on distinct promoters [40,41]. PRMT-1-mediated asymmetrical

dimethylation of R109 increases the binding of E2F-1 to apoptotic target gene promoters [40]. Symmetrical dimethylation of R111 and R113 by PRMT5 corresponds to E2F-1 presence on growth promoting target genes [40,41]. In this study, we found PRMT-1-catalyzed asymmetrical dimethylation of R484/516 enhance the binding of SKN-1 on phase II detoxification gene promoters. Whether symmetrical dimethylation of arginines occurs on SKN-1, and has functions in other cellular process is worth exploring in the further.

In summary, we elucidate a conserved modification, the asymmetrical arginine dimethylation catalyzed by PRMT-1, on NRF/CNC family during antioxidative response from worms to human cells. These findings have important implications for studying the mechanism by which NRF/CNC transcription factors function in the human diseases related to oxidative stress.

Acknowledgments

This work was supported by the grants from the National Natural Science Foundation of China (Grant Numbers 31871282 to X.L., 31770825, 31571317 to J.L., and 31571339 to X.B.), the Jilin Provincial Science & Technology Department (Grant Number 20190201183JC), and the Development Project of Science and Technology of Jilin Province (Grant Number Y831141001). We thank Prof. T. Keith Blackwell (Research Division, Joslin Diabetes Center) for providing SKN-1B/C::GFP plasmids. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

Author contributions

Xiaoxue Li, Hongyuan Li and Jun Lu conceived and designed the experiments. Hongyuan Li, Liangping Su and Dan Wang performed the experiments. Xin Liu and Xin Su, Hongmei Li and Dan Wang prepared the compounds used in this study. Hongyuan Li, Liangping Su and Xiaoxue Li analyzed the data and figures. Xiaoxue Li, Hongyuan Li, and Liangping Su wrote the paper. Baiqu Huang, Xueqing Ba and Yu Zhang revised the paper. All the authors discussed the results and commented on the manuscript.

Declarations of interest

None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2019.101111.

References

[1] R. Thanan, S. Oikawa, Y. Hiraku, S. Ohnishi, N. Ma, S. Pinlaor, P. Yongvanit, S. Kawanishi, M. Murata, Oxidative stress and its significant roles in neurodegenerative diseases and cancer, Int. J. Mol. Sci. 16 (2014) 193–217.

[2] H. Cui, Y. Kong, H. Zhang, Oxidative stress, mitochondrial dysfunction, and aging, J. Signal Transduct. 2012 (2012) 646354.

[3] M. Brownlee, Biochemistry and molecular cell biology of diabetic complications, Nature 414 (2001) 813–820.

[4] G.P. Sykiotis, D. Bohmann, Stress-activated cap'n'collar transcription factors in aging and human disease, Sci. Signal. 3 (2010) re3.

[5] T.W. Kensler, N. Wakabayashi, S. Biswal, Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway, Annu. Rev. Pharmacol. Toxicol. 47 (2007) 89–116.

[6] M. McMahon, K. Itoh, M. Yamamoto, S.A. Chanas, C.J. Henderson, L.I. McLellan, C.R. Wolf, C. Cavin, J.D. Hayes, The Cap'n'Collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes, Cancer Res. 61 (2001) 3299–3307.

[7] T.K. Blackwell, M.J. Steinbaugh, J.M. Hourihan, C.Y. Ewald, M. Isik, SKN-1/Nrf, stress responses, and aging in Caenorhabditis elegans, Free Radic. Biol. Med. 88 (2015) 290–301.

[8] J.H. An, T.K. Blackwell, SKN-1 links C. elegans mesendodermal specification to a conserved oxidative stress response, Genes Dev. 17 (2003) 1882–1893.

[9] A.K. Walker, R. See, C. Batchelder, T. Kophengnavong, J.T. Gronniger, Y. Shi, T.K. Blackwell, A conserved transcription motif suggesting functional parallels between Caenorhabditis elegans SKN-1 and Cap'n'Collar-related basic leucine zipper proteins, J. Biol. Chem. 275 (2000) 22166–22171.

[10] J.H. An, K. Vranas, M. Lucke, H. Inoue, N. Hisamoto, K. Matsumoto, T.K. Blackwell, Regulation of the Caenorhabditis elegans oxidative stress defense protein SKN-1 by glycogen synthase kinase-3, Proc. Natl. Acad. Sci. USA 102 (2005) 16275–16280.

[11] H. Inoue, N. Hisamoto, J.H. An, R.P. Oliveira, E. Nishida, T.K. Blackwell, K. Matsumoto, The C. elegans p38 MAPK pathway regulates nuclear localization of the transcription factor SKN-1 in oxidative stress response, Genes Dev. 19 (2005) 2278–2283.

[12] Y. Kawai, L. Garduno, M. Theodore, J. Yang, I.J. Arinze, Acetylation-deacetylation of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) regulates its transcriptional activity and nucleocytoplasmic localization, J. Biol. Chem. 286 (2011) 7629–7640.

[13] Z. Sun, Y.E. Chin, D.D. Zhang, Acetylation of Nrf2 by p300/CBP augments promoter-specific DNA binding of Nrf2 during the antioxidant response, Mol. Cell. Biol. 29 (2009) 2658–2672.

[14] Z. Sun, Z. Huang, D.D. Zhang, Phosphorylation of Nrf2 at multiple sites by MAP kinases has a limited contribution in modulating the Nrf2-dependent antioxidant response, PLoS One 4 (2009) e6588.

[15] M. Salazar, A.I. Rojo, D. Velasco, R.M. de Sagarra, A. Cuadrado, Glycogen synthase kinase-3beta inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2, J. Biol. Chem. 281 (2006) 14841–14851.

[16] H.C. Huang, T. Nguyen, C.B. Pickett, Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription, J. Biol. Chem. 277 (2002) 42769–42774.

[17] S.B. Cullinan, J.D. Gordan, J. Jin, J.W. Harper, J.A. Diehl, The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase, Mol. Cell. Biol. 24 (2004) 8477–8486.

[18] J.M. Tullet, M. Hertweck, J.H. An, J. Baker, J.Y. Hwang, S. Liu, R.P. Oliveira, R. Baumeister, T.K. Blackwell, Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in C. elegans, Cell 132 (2008) 1025–1038.

[19] H. Li, X. Liu, D. Wang, L. Su, T. Zhao, Z. Li, C. Lin, Y. Zhang, B. Huang, J. Lu, X. Li, O-GlcNAcylation of SKN-1 modulates the lifespan and oxidative stress resistance in Caenorhabditis elegans, Sci. Rep. 7 (2017) 43601.

[20] M.T. Bedford, S.G. Clarke, Protein arginine methylation in mammals: who, what, and why, Mol. Cell 33 (2009) 1–13.

[21] T.B. Nicholson, T. Chen, S. Richard, The physiological and pathophysiological role of PRMT1-mediated protein arginine methylation, Pharmacol. Res. 60 (2009) 466–474.

[22] B.T. Schurter, F. Blanchet, O. Acuto, Protein arginine methylation: a new frontier in T cell signal transduction, Adv. Exp. Med. Biol. 584 (2006) 189–206.

[23] S. Pahlich, R.P. Zakaryan, H. Gehring, Protein arginine methylation: cellular functions and methods of analysis, Biochim. Biophys. Acta 1764 (2006) 1890–1903.

[24] M.T. Bedford, S. Richard, Arginine methylation an emerging regulator of protein function, Mol. Cell 18 (2005) 263–272.

[25] F. Herrmann, P. Pably, C. Eckerich, M.T. Bedford, F.O. Fackelmayer, Human protein arginine methyltransferases in vivo–distinct properties of eight canonical members of the PRMT family, J. Cell Sci. 122 (2009) 667–677.

[26] K.A. Mowen, M. David, Analysis of protein arginine methylation and protein arginine-methyltransferase activity, Sci. STKE 2001 (2001) l1.

[27] X. Liu, H. Li, L. Liu, Y. Lu, Y. Gao, P. Geng, X. Li, B. Huang, Y. Zhang, J. Lu, Methylation of arginine by PRMT1 regulates Nrf2 transcriptional activity during the antioxidative response, Biochim. Et. Biophys. Acta 1863 (2016) 2093–2103.

[28] S. Kimura, S. Sawatsubashi, S. Ito, A. Kouzmenko, E. Suzuki, Y. Zhao, K. Yamagata, M. Tanabe, T. Ueda, S. Fujiyama, T. Murata, H. Matsukawa, K. Takeyama, N. Yaegashi, S. Kato, Drosophila arginine methyltransferase 1 (DART1) is an ecdysone receptor co-repressor, Biochem. Biophys. Res. Commun. 371 (2008) 889–893.

[29] M.R. Pawlak, C.A. Scherer, J. Chen, M.J. Roshon, H.E. Ruley, Arginine N-methyltransferase 1 is required for early postimplantation mouse development, but cells deficient in the enzyme are viable, Mol. Cell. Biol. 20 (2000) 4859–4869.

[30] Y. Takahashi, H. Daitoku, K. Hirota, H. Tamiya, A. Yokoyama, K. Kako, Y. Nagashima, A. Nakamura, T. Shimada, S. Watanabe, K. Yamagata, K. Yasuda, N. Ishii, A. Fukamizu, Asymmetric arginine dimethylation determines life span in C. elegans by regulating forkhead transcription factor DAF-16, Cell Metab. 13 (2011) 505–516.

[31] D. Hoogewijs, K. Houthoofd, F. Matthijssens, J. Vandesompele, J.R. Vanfleteren, Selection and validation of a set of reliable reference genes for quantitative sod gene expression analysis in C. elegans, BMC Mol. Biol. 9 (2008) 9.

[32] A. Mukhopadhyay, B. Deplancke, A.J. Walhout, H.A. Tissenbaum, Chromatin immunoprecipitation (ChIP) coupled to detection by quantitative real-time PCR to study transcription factor binding to DNA in Caenorhabditis elegans, Nat. Protoc. 3 (2008) 698–709.

[33] S. Ayyadevara, R. Alla, J.J. Thaden, R.J. Shmookler Reis, Remarkable longevity and stress resistance of nematode PI3K-null mutants, Aging Cell 7 (2008) 13–22.

[34] E. de Castro, S. Hegi de Castro, T.E. Johnson, Isolation of long-lived mutants in Caenorhabditis elegans using selection for resistance to juglone, Free Radic. Biol. Med. 37 (2004) 139–145.

[35] K.I. Zhou, Z. Pincus, F.J. Slack, Longevity and stress in Caenorhabditis elegans, Aging 3 (2011) 733–753.

[36] L. Sha, H. Daitoku, S. Araoi, Y. Kaneko, Y. Takahashi, K. Kako, A. Fukamizu,

Asymmetric arginine dimethylation modulates mitochondrial energy metabolism and homeostasis in Caenorhabditis elegans, Mol. Cell. Biol. 37 (2017).

[37] B. Meissner, T. Rogalski, R. Viveiros, A. Warner, L. Plastino, A. Lorch, L. Granger, L. Segalat, D.G. Moerman, Determining the sub-cellular localization of proteins within Caenorhabditis elegans body wall muscle, PLoS One 6 (2011) e19937.

[38] L. Etheve, J. Martin, R. Lavery, Dynamics and recognition within a protein-DNA complex: a molecular dynamics study of the SKN-1/DNA interaction, Nucleic Acids Res. 44 (2016) 1440–1448.

[39] S.M. Carr, A. Poppy Roworth, C. Chan, N.B. La Thangue, Post-translational control of transcription factors: methylation ranks highly, FEBS J. 282 (2015) 4450–4465.

[40] S. Zheng, J. Moehlenbrink, Y.C. Lu, L.P. Zalmas, C.A. Sagum, S. Carr, J.F. McGouran, L. Alexander, O. Fedorov, S. Munro, B. Kessler, M.T. Bedford, Q. Yu, N.B. La Thangue, Arginine methylation-dependent reader-writer interplay governs growth control by E2F-1, Mol. Cell 52 (2013) 37–51.

[41] E.C. Cho, S. Zheng, S. Munro, G. Liu, S.M. Carr, J. Moehlenbrink, Y.C. Lu, L. Stimson, O. Khan, R. Konietzny, J. McGouran, A.S. Coutts, B. Kessler, D.J. Kerr, N.B. Thangue, Arginine methylation controls growth regulation by E2F-1, EMBO J. 31 (2012) 1785–1797.