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Stabilization of RNT-1 Protein, Runt-related Transcription Factor (RUNX) Protein Homolog of *Caenorhabditis elegans*, by Oxidative Stress through Mitogen-activated Protein Kinase Pathway*[S]

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Background: RUNX proteins are important for development, but not much is known about the functions of RUNX after development.
Results: RNT-1, the nematode RUNX homolog, was stabilized by oxidative stress through a p38 MAP kinase for proper response to oxidative stress.
Conclusion: RNT-1 plays a role in defensive responses to oxidative stress.
Significance: We identified a new role for a RUNX protein in a postdevelopmental process.

RUNX proteins are evolutionarily conserved transcription factors known to be involved in various developmental processes. Here we report a new role for a RUNX protein: a role in stress response. We show that RNT-1, the *Caenorhabditis elegans* RUNX homolog, is constantly produced and degraded by the ubiquitination-proteasome pathway in the intestine of the nematode. RNT-1 was rapidly stabilized by oxidative stress, and the *rnt-1*-mutant animals were more sensitive to oxidative stress, indicating that rapid RNT-1 stabilization is a defense response against the oxidative stress. The MAP kinase pathway is required for RNT-1 stabilization, and RNT-1 was phosphorylated by SEK-1/PMK-1 *in vitro*. ChIP-sequencing analysis revealed a feedback loop mechanism of the MAP kinase pathway by the VHP-1 phosphatase in the RNT-1-mediated oxidative stress response. We propose that *rnt-1* is regulated at the protein level for its role in the immediate response to environmental challenges in the intestine.

RUNX[3] family proteins, evolutionarily well conserved transcription factors, have pivotal roles in various aspects of development such as hematopoiesis, chondrogenesis, neurogenesis, and cancer development (1–4). The effects of RUNX deregulation in various developmental processes have been studied. Deletion or null mutation as well as overexpression of the RUNX family caused aberrant phenotypes such as acute myelogenous leukemia, cleidocranial dysplasia, and gastric adenocarcinoma, suggesting that RUNX exerts its effects in a dose-dependent manner (5). In addition, diverse cellular signaling events that modify both the transcriptional and posttranscriptional status of the RUNX genes affect the amount and the activity of RUNX proteins in response to developmental or environmental cues. Transcriptional regulation, such as chromatin remodeling (6) and DNA methylation (7–10), and post-transcriptional regulation, such as acetylation (11, 12), ubiquitination (13–15), and phosphorylation (16, 17), have been reported as the regulation mechanisms of RUNX activity.

In *Caenorhabditis elegans*, only one functional RUNX gene, *rnt-1*, was identified (18). Mutations in *rnt-1* caused severe morphological defects induced by improper development of the hypodermis and the intestine (19). In *C. elegans*, RNT-1 is also involved in cell fate commitment in the seam cell division, which is similar to the functions of mammalian RUNXs (20). The function of RNT-1 in cell cycle regulation is explained by its genetic interaction with *cki-1*, a cyclin-dependent kinase inhibitor. Transcriptional regulation of *rnt-1* has been confirmed in a previous study (21). From nematodes to humans, the biological functions of RUNX proteins have been limited to developmental processes such as normal development or carcinogenesis, and their functions, aside from those in development, have not been fully studied. In this study, we wanted to pursue this issue using *C. elegans* as a model organism.

Here, we report that RNT-1, a RUNX family protein, has a new role in stress response. We show that the level of RNT-1 proteins was up-regulated by oxidative and osmotic stress via stabilization of the proteins, which are otherwise degraded by proteasome-mediated proteolysis. The stabilization of RNT-1 proteins was mediated by the p38 MAP kinase pathway. We also show that *vhp-1*, one of the target genes of stabilized RNT-1, was transcriptionally activated by RNT-1 and formed a negative feedback loop by modulating the PMK-1 pathway for appropriate oxidative stress response.

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[S] This article contains supplemental Tables 1 and 2, Figs. 1–6, and references.
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[3] The abbreviation used are: RUNX, runt-related transcription factor; MAPK, Mitogen-activated Protein Kinase; NGM, nematode growth medium.

EXPERIMENTAL PROCEDURES

Worm Strains and Culture—*C. elegans* strains of the genotype of wild-type N2 Bristol and *rnt-1*(ok351) was obtained from the *C. elegans* Genome Center (Minneapolis, MN), and the *rnt-1*(tm388) and *rnt-1*(tm491) strain was obtained from the National Bioresources Project (Tokyo, Japan). Worms were grown at 20 °C on nematode growth media (NGM) by a standard method (22).

Microinjection and Microscopy—Microinjection of DNA into the gonads of the adult hermaphrodites was carried out according to a standard protocol (23). The pPD-run-FL construct (19), which bears sequences of the full-length *rnt-1* promoter and RNT-1 protein-fused GFP, was used for expression analysis. For intestine-specific rescue of the defect in the *rnt-1* mutant, 2 kb of *act-5* promoter at and cDNA of *rnt-1* were cloned to the pPD95.77 vector. The pRF4, which contains the dominant *rol-6* (*su1006*) gene, was used as an injection marker at the concentration of 100 μg/ml (24). All plasmids were injected at the concentration of 100 μg/ml. Plasmid DNAs used for injection were extracted with the Qiagen plasmid midi kit (catalog number 12145). To see the expression pattern of transgenes, the transgenic lines were paralyzed with 5 mM levamisole and mounted on 5% agar pads, and the fluorescence was observed using an Axioplan 2 microscope.

Feeding RNAi Method—For *pmk-1* RNAi, we used the HT115 *Escherichia coli* strain containing the full-length *pmk-1* cDNA in the pPD129.36 (L4440) vector. The clone was confirmed by sequencing. For *ubq-1, uba-1, math-33, sek-1, nsy-1,* and *vhp-1* RNAi, we used an RNAi library purchased from MRC (Cambridge, UK). We started RNAi from the L4 stage for *pmk-1, sek-1,* and *nsy-1* RNAi and examined the phenotypes of the L4 progeny. For *ubq-1* and *uba-1* RNAi, whose RNAi knockdown caused embryonic lethality, we provided L1 larvae with RNAi bacteria and observed the phenotypes when they reached adulthood. For *vhp-1*, we provided L2 larvae with RNAi bacteria and observed the phenotypes when they reached adulthood. Worms were fed by each RNAi *E. coli* strain on NGM plates containing 1 mM isopropyl 1-thio-β-D-galactopyranoside.

In Situ Hybridization—Detection of *rnt-1* transcripts in embryos, larvae, and adult worms was performed as described in Ref. 25. Digoxigenin-dideoxy UTP-labeled oligonucleotide was synthesized, followed by the protocol (Bionex, Korea). We used worms containing pPD-run-FL and pRF4 to detect *rnt-1* RNA.

Proteasome Inhibitor Treatment—To test whether the proteasome pathway degrades RNT-1, we treated the worms with the commonly used proteasome inhibitor MG132 (Sigma). The MG132 experiments were performed as described previously, with some modifications (26–28). Briefly, the transgenic animals containing the pPD-run-FL reporter construct were grown on NGM plates. Then, we washed off the plates with M9 (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 80 mM NaCl, 1 mM $MgSO_4$) medium and collected into a 1.5-ml microcentrifuge tube. MG132 was added at a final concentration of 50 μM, after which the animals were incubated for 6 h at room temperature on a Nutator mixer and then washed with M9. To see the difference between MG132-treated worms and M9-treated control worms, both lines were mounted on 5% agar pads, and the fluorescence was observed using an Axioplan2 microscope. To perform the Western blot analysis, worms were grown on 100-mm NGM lite media plates and harvested with M9 buffer. The harvested worms were vigorously shaken in 100 ml of M9 with or without 50 μM MG132 for 6 h.

Immunoprecipitation—To show that RNT-1 is ubiquitinated, plasmids harboring a cDNA of RNT-1 under the CMV promoter and an ubiquitin gene, respectively, were transfected to the 293T cell line. After 24-h incubation, we treated the cells with MG132 for 2 h and harvested them. The following immunoprecipitation and Western blotting were performed with a standard protocol.

Stress Assay—To examine whether RNT-1::GFP was stabilized by oxidative stress and osmotic stress, *rnt-1::gfp* worms were applied to M9 solution containing 0.1 M paraquat, 10 mM *t*-butyl peroxide, 0.3 M NaCl, and 625 mM sucrose, respectively. After 0, 30, 60, and 240 min, we harvested worms with M9 and then observed them with an Axioplan2 microscope. To give heat shock, we put worms into a 30 °C incubator for 30 min on NGM plates. For cold shock, worms were incubated at 4 °C for 30 min. Hypoxia was induced by soaking the worms for 1 h in M9 solution. Worms were irradiated with UV light (360 nm, 340 W/cm^2, XL-1000 UV Crosslinker, Spectronics Co.) for 1 min. After each stress, worms were mounted on 5% agar pads, and the fluorescence was observed using an Axioplan2 microscope (Zeiss). Images were taken by an AxioCam (Zeiss) camera. To analyze the time course expression level of RNT-1 fused to GFP in the intestine, we only measured its fluorescence intestine in the int1 cells for consistency.

To test whether *rnt-1* mutant alleles were sensitive to paraquat, we performed a paraquat lethality test. We synchronized N2 and *rnt-1* worms and harvested them when they reached the L4 stage. All L4-stage worms were washed off with M9 buffer and put on empty NGM plates. By using 0.4 M paraquat solution, 50 worms were picked up with a mouth pipette and moved into an empty 24-well plate (29). More 0.4 M paraquat solution was dropped on the worms to avoid dehydration. After 5 min of exposure to paraquat, thrashing worms were counted. Each assay was repeated more than three times, and all of them were carried out at 22 °C.

To test the relationship between the MAP kinase pathway and RNT-1::GFP stabilization in oxidative stress, we synchronized control *rnt-1::gfp* worms and *pmk-1, sek-1, nsy-1,* and *vhp-1* RNAi-treated *rnt-1::gfp* worms to L4 stage or young adulthood. L4 or young adult worms were harvested and washed off with M9 buffer. We applied 0.1 M paraquat to the animals, and 50–70 worms were transferred to an empty 24-well plate with a mouth pipette.

To test whether N2 and *rnt-1(tm491)* mutants fed with L4440 or *vhp-1* RNAi bacteria were sensitive to paraquat, we performed a paraquat lethality test. We synchronized N2 and *rnt-1(tm491)* strains, fed them with L4440 or *vhp-1* RNAi bacteria from the L2 stage, and harvested them when they reached the L4 stage. All L4-stage worms were washed off with M9 buffer, and 50–60 worms were applied to M9 solution containing 0.1 M paraquat at room temperature (22 °C). Dead worms were confirmed by no movement after touching their bodies.

In Vitro Kinase Assay—To test whether *pmk-1* kinase activity phosphorylated RNT-1, we performed an *in vitro* kinase assay. FLAG-tagged PMK-1 and/or MYC-tagged SEK-1 was purified by anti-FLAG and/or anti-MYC-agarose bead from HEK293T cell lysate. Various GST-tagged constructs of RNT-1, PMK-1, and/or SEK-1 immunoprecipitates were incubated with [γ-^{32}P]ATP (10 μCi) in kinase buffer (200 mM HEPES (pH7.3), 200 mM $MgCl_2$, 200 mM $MnCl_2$, 10 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 10 mM DTT, 4 mM PMSF) for 20 min at 30 °C (31). The reaction was stopped by the addition of sample buffer. Phosphorylated RNT-1 was subjected to 9% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography (Fuji, BAS 2000).

In Vitro Site-directed Mutagenesis—To make point mutations in putative phosphorylation sites of RNT-1, each plasmid was mutated by PCR-based mutagenesis with a QuikChange site-directed mutagenesis kit provided by Stratagene.

Chromatin Immunoprecipitation and Further Analyses—We followed the protocol described previously to carry out the ChIP experiments (32). Briefly, mixed-stage RNT-1::GFP transgenic worms were grown on NGM lite plates and harvested. After cross-linking the worm extract, the pellet was washed and resuspended in HEPES lysis buffer. We sonicated the pellet more than eight times. After centrifuging the pellet, supernatant was precleared with salmon sperm DNA/protein A-agarose and incubated with anti-GFP antibody at 4 °C overnight. To identify effector genes downstream of RNT-1 in response to oxidative stress, we performed ChIP-sequencing experiments in the presence or absence of oxidative stress. DNA samples from the ChIP assay were sequenced by next-generation sequencing in the laboratory of one of the authors (Y. J.K.) and Macrogen (Korea), and all data were analyzed by Macrogen (Korea). We compared data sets of the absence and presence of oxidative stress and selected nine genes on the basis of the existence of conserved *rnt-1* binding sites and confirmation by real-time quantitative PCR analysis. Real-time quantitative PCR was carried out with TaKaRa SYBR premix Ex Taq in Applied Biosystem Prism 7300 and Bio-Rad iQTM SYBR Green Supermix in iQ5 as described in the manufacturer's manual.

RNA Preparation and Quantitative Real-time PCR Analysis—Total RNA was isolated with TRIzol reagent (Invitrogen) with the freeze-thaw method according to standard protocol of the manufacturer's manual. cDNA was synthesized with RevertAid M-MuLV reverse transcriptase (Fermentas, Canada) using oligo(dT) primer and subjected to PCR amplification.

PCR Primers —Sequences of the primers used in this study are listed in supplemental Table 1.

RESULTS

Proteasome-mediated Degradation of RNT-1 in the Intestine of C. elegans—RNT-1 expression in the nematode is tightly regulated in terms of developmental timing and space because GFP reporter assays showed that RNT-1 is expressed in the hypodermis and the intestine from the embryonic stage up to larval stages and that its expression disappears in the adult stage (19, 21). To determine the regulatory mechanism underlying this specific expression, we examined the RNA and protein levels of *rnt-1* during development. Interestingly, the *in situ* hybridization analysis showed that *rnt-1* transcription still occurred in the intestine at the adult stage (Fig. 1*A*), whereas GFP-fused RNT-1 proteins were undetectable (*B*), indicating that RNT-1 regulation occurs at the posttranscriptional level. Because the *rnt-1* transcript was still abundant in the intestine, we examined whether RNT-1 was regulated at the level of protein stability. When the nematodes were treated with MG132, a proteasome inhibitor, the GFP fused RNT-1 protein was stabilized only in the intestine at all developmental stages, including the adult stage (Fig. 1*B*), indicating that the proteasome continuously degrades RNT-1 in the intestine. We confirmed this result by Western blot analysis (supplemental Fig. 1). Consistent with this, RNAi knockdown of *ubq-1*, a polyubiquitin gene, and *uba-1*, the E1 gene, resulted in higher stability of RNT-1 in the intestine (Fig. 1*B*). In addition, RNAi of a ubiquitin-specific hydrolase, *math-33*, resulted in the stabilization of RNT-1. However, knockdown of C18E3.2, *swsn-2.1, swsn-2.2*, T24G10.2, and R07B7.2, which are annotated as homologs of MDM2, an E3 ligase of RUNX3 (15), did not cause stabilization of RNT-1 proteins (data not shown), suggesting that an E3 ligase different from MDM2 may act for RNT-1 in *C. elegans*. To confirm that RNT-1 is indeed ubiquitinated, we expressed RNT-1 under the CMV promoter in 293T cells together with an ubiquitin construct (Fig. 1*C*). Ubiquitination of RNT-1 was detected, and the treatment of MG132 increased the level of ubiquitinated RNT-1. These findings indicate that proteasome-mediated degradation is a main process of RNT-1 stability control in the intestine by accelerating its turnover rate.

Oxidative and Osmotic Stresses Stabilize RNT-1 in the Intestine—A possible biological implication of RNT-1 regulation at the protein level instead of the transcriptional level was that RNT-1 could be involved in a rapid response to environmental changes, which might require rapid changes in cell physiology. To test this hypothesis, we examined whether RNT-1 in the intestine was stabilized by external stress conditions. Among the various stresses, oxidative stress induced by paraquat and *t*-butyl peroxide and osmotic stress induced by high concentration of NaCl and sucrose rapidly stabilized RNT-1 in the intestine, which was not affected by heat shock, cold shock, hypoxia, and UV light (Fig. 2*A*). We could detect the GFP signal from stabilized RNT-1 proteins within 30 min after treating oxidative stress (Fig. 2, *B* and *C*). In the oxidative stress condition, stabilized RNT-1 was gradually decreased after 30 min and totally diminished within 4 h. We confirmed that the oxidative stress conditions did not increase the transcript level of *rnt-1* (supplemental Fig. 2), again confirming that the protein level, not the transcription level, was affected by the stress conditions. These results raised the possibility that gaining of RNT-1 stability in the intestine might reflect its role in the acute stress response in the intestine.

RNT-1 Is Involved in the Oxidative Stress Response—To test whether RNT-1 was involved in the defensive mechanism against oxidative stress, we examined the susceptibility of *rnt-1* mutant animals to paraquat treatment. After being exposed to 400 mM paraquat for 5 min, approximately 30% of the wild-type worms were still alive, but only 10% of *rnt-1* mutant worms survived (Fig. 3*A*). The defective response to oxidative stress in *rnt-1* mutant alleles was rescued with full-length RNT-1

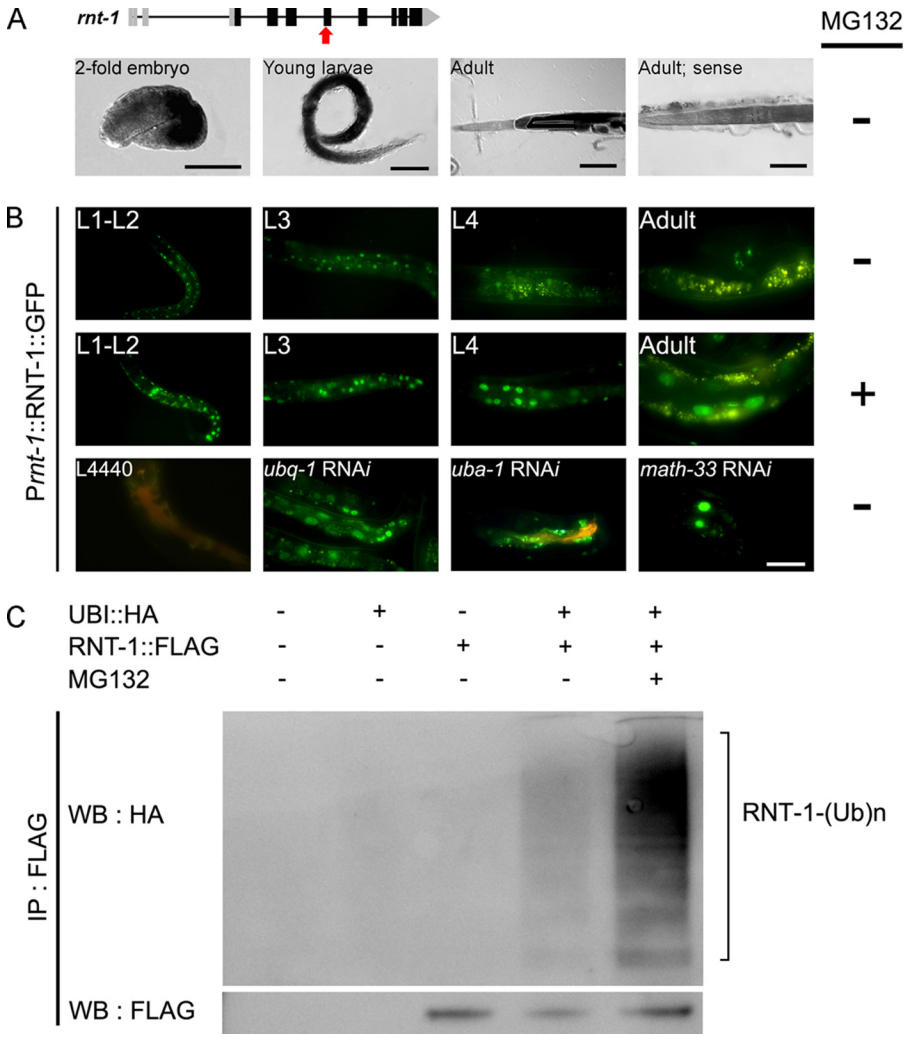

FIGURE 1. **Continuous degradation of RNT-1 proteins in the intestine.** *A*, the *upper panel* shows the genomic structure of the *rnt-1* gene, and the *bottom panels* show the *in situ* hybridization results of *rnt-1* at different developmental stages. The *white line* and the *white dotted line* in the adult indicate the intestine and gonadal arm, respectively. The *red arrow* in the *upper panel* represents the location of the *in situ* probe of *rnt-1*. A sense probe was used as the negative control. *B*, the *top two rows* show GFP expression in N2 animals containing the RNT-1::GFP transgene in the absence or presence of MG132, respectively. The *third row* shows the results of knockdown of *ubq-1* (polyubiquitin), *uba-1* (E1), and *math-33* (ubiquitin-specific hydrolase) at the young adult stage. L4440 was used as s negative control. *Scale bars* = 50 μm. *C*, ubiquitination of RNT-1 in 293T cells. The ubiquitinated FLAG-tagged RNT-1 was detected in *lanes 4* and *5*. Treatment of MG132 increased ubiquitinated RNT-1 (*lane 5*).*WB*, Western blotting; *IP*, immunoprecipitation.

expression under its own promoter (supplemental Fig. 3). We were able to rescue the defective oxidative stress response by expressing RNT-1 under an intestine-specific promoter, *act-5* (supplemental Fig. 3), suggesting that RNT-1 in the intestine is necessary for the stress response. Taken together, RNT-1 is required for a defensive response against oxidative stress.

The MAPK Pathway Is Responsible for Regulating RNT-1 in the Stress Response—The p38 MAP kinase pathway is responsible for various stress responses in *C. elegans* (30, 33). To examine whether the p38 MAP kinase pathway is involved in the stabilization of RNT-1 in the presence of oxidative stress, we examined the knockdown effects of *nsy-1*, *sek-1*, and *pmk-1*, which encode MAP3K, MAP2K, and MAPK, respectively. We found that RNAi of any of these genes prevented accumulation of RNT-1 proteins in the intestine after exposure to each stress (Fig. 3*B*). We excluded the possibility of the involvement of other MAP kinases such as *pmk-2*, *pmk-3* (p38), *jnk-1* (JNK), and *mpk-1* (ERK) (data not shown). However, RNAi of *kgb-1* or *mek-1* partially diminished the intensity of the stabilized GFP signal (supplemental Fig. 4), suggesting that these genes may be partially involved. Using an *in vitro* phosphorylation system, we found that purified SEK-1 and PMK-1, but not SEK-1 or PMK-1 alone, were able to phosphorylate RNT-1 (Fig. 4*A*). We also confirmed that the C-terminal region of the RNT-1 protein, which bears multiple serine/threonine sites, is responsible for phosphorylation-mediated modification by SEK-1/PMK-1, whereas the Runt domain was dispensable (Fig. 4*B*). These data suggest that p38 MAPK directly phosphorylates RNT-1 so that RNT-1 can be stabilized. To identify the phosphorylation sites of RNT-1, we first tested whether serine 255 is phosphorylated because p38 MAPK has a conserved phosphorylation site of Pro-*X*-Ser/Thr-Pro (Fig. 4*C* and supplemental Fig. 5*A*). We identified that the polypeptide containing the 251st to the 301st amino acid of RNT-1 was phosphorylated and that the mutation in serine 255 to alanine dramatically decreased the phosphorylation level. We also found that the polypeptide contain-

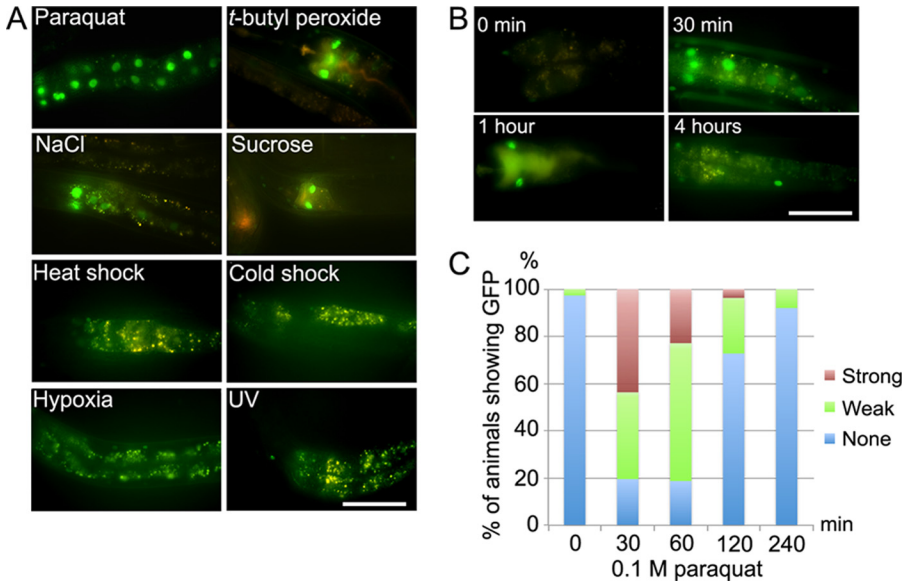

FIGURE 2. **Oxidative and osmotic stresses stabilize RNT-1::GFP in the intestine.** *A*, oxidative stress (paraquat, *t*-butyl peroxide) and osmotic stress (NaCl, sucrose) stabilize RNT-1::GFP in wild-type animals expressing a RNT-1::GFP transgene. Heat shock, cold shock, hypoxia, or UV light did not affect the stability of RNT-1. *B*, time course changes in the RNT-1::GFP abundance after treating paraquat. Late L4 or young adults were used. *C*, quantification of the animals for their levels of stabilized RNT-1::GFP under oxidative stress conditions. The expressions of GFP in int1 cells were analyzed. *Scale bars* = 50 μm.

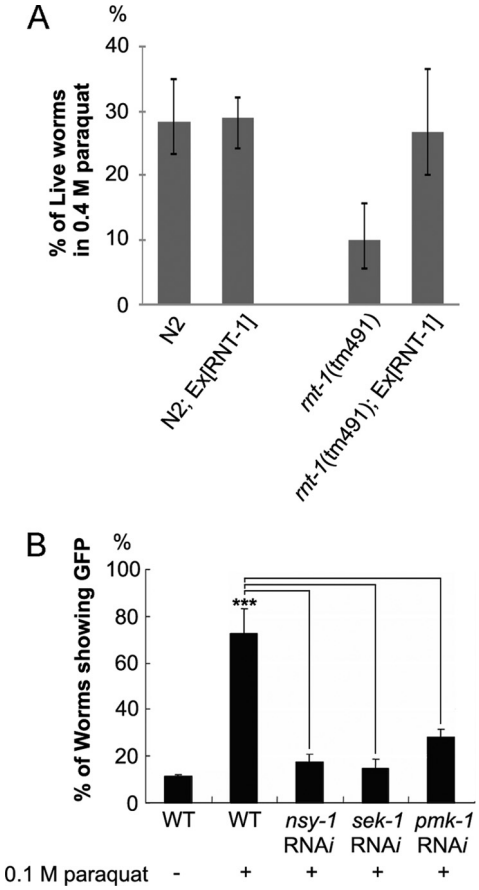

FIGURE 3. **RNT-1 is required for response to oxidative stress.** *A*, survival rates of N2 and *rnt-1(tm491)* animals after 5-min exposure to 0.4 M paraquat for acute oxidative stress. *B*, stabilization of RNT-1 in the intestine by oxidative stress is dependent on *nsy-1*, *sek-1*, and *pmk-1*. ***, $p < 0.001$.

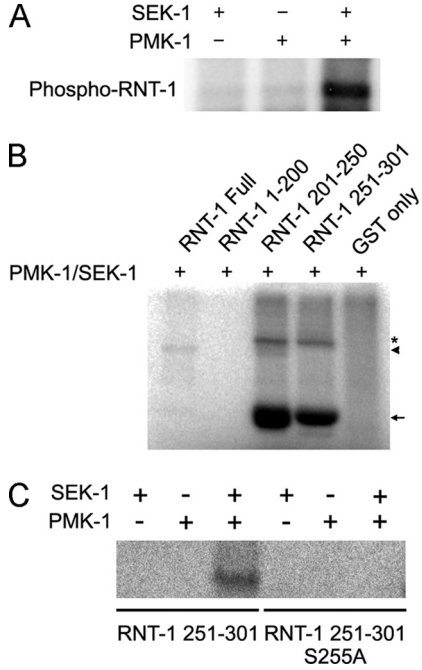

FIGURE 4. **Purified PMK-1 can directly phosphorylate the C-terminal regions of RNT-1 *in vitro*.** *A*, the result of the *in vitro* kinase assay of RNT-1 with SEK-1/PMK-1 kinases. *B*, domain studies of RNT-1 using GST-fused polypeptides containing 1–200, 201–250, or 251–301 amino acids. The *asterisk* is a nonspecific signal or a dimerized protein signal. The *arrowhead* is the signal of RNT-1 full sequence. The *arrow* is the GST-partial RNT-1 fusion protein signal. *C*, the result of the *in vitro* kinase assay with wild-type and the mutation of serine 255 to alanine of RNT-1.

ing the 201st to the 251st amino acid of RNT-1 was phosphorylated (Fig. 4*B*), but we failed to find the phosphorylation site, although we tried three putative MAPK phosphorylation sites (supplemental Fig. 5*B*). These data raise the possibility of the existence of other phosphorylation site(s).

vhp-1, a Phosphatase of the MAP Kinase Pathway, Is a Transcriptional Target of RNT-1—As a first attempt to identify downstream target genes of RNT-1 in response to stresses, we performed ChIP-sequencing experiments in the presence or absence of oxidative stress. Among the candidate target genes

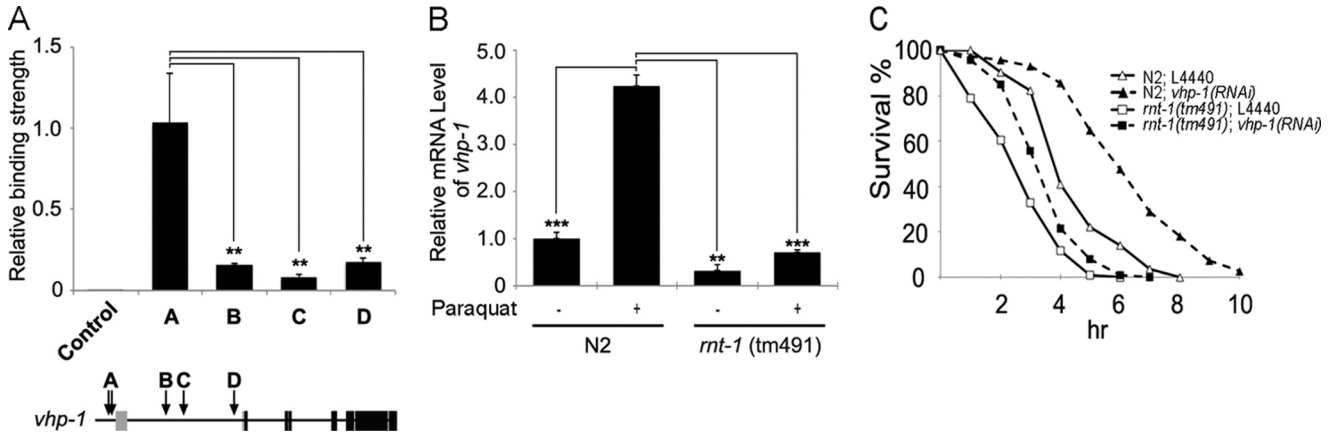

FIGURE 5. ***vhp-1* is a target of RNT-1 in oxidative stress response.** *A*, chromatin immunoprecipitation quantitative PCR analysis on the promoter of *vhp-1*. The *arrows* indicate the locations of the putative RNT-1 binding sequences. In *A*, two putative RNT-1 binding sequences are closely located. *B*, relative mRNA levels of *vhp-1* in the absence or presence of 0.1 M paraquat in wild-type N2 and *rnt-1* mutant animals. *C*, effects of *vhp-1* RNAi on the survival rates of N2 and *rnt-1*(tm491) in M9 buffer containing 0.1 M paraquat. Additional experiments are presented in supplemental Fig. 4. **, $p < 0.01$; ***, $p < 0.001$.

(listed in supplemental Table 2), we focused on *vhp-1* because this gene encodes a phosphatase of MAP kinase. *vhp-1* has been reported to function in various stress responses that are related to the MAP kinase pathway (34–37). All five conserved *rnt-1* binding sites are at the promoter of *vhp-1*. We tested where RNT-1 actually binds by ChIP-quantitative PCR analysis. We found that the binding site between 9683 and 9731 nucleotides upstream of the translational start site of *vhp-1*, which bears two conserved RNT-1 binding sequences (marked *A* in Fig. 5*A*), was responsible for RNT-1 binding *in vivo*. Next, we checked whether transcription of *vhp-1* was regulated by RNT-1 in the oxidative stress condition (Fig. 5*B*). In wild-type animals, the transcription of *vhp-1* was increased by paraquat treatment. In *rnt-1*-mutant animals, however, the transcription level of *vhp-1* was dramatically decreased, both in the presence and absence of paraquat treatment. We also tested whether the disruption of *vhp-1* gene activity could affect the sensitivity to oxidative stress (Fig. 5*C* and supplemental Fig. 6). Knockdown of *vhp-1* by RNAi increased the resistance to 100 mM paraquat in the wild-type background but only partially in the *rnt-1* mutant background. The observation that RNAi of *vhp-1* still caused a partially increased resistance in the *rnt-1* mutant background suggests that *vhp-1* is involved in *rnt-1*-independent response pathways. Transcription of *vhp-1* was not activated by osmotic stress, and knockdown of *vhp-1* did not show significant resistance to high osmolality (data not shown), suggesting that *vhp-1* functions as a negative regulator of the oxidative stress response but not the osmotic stress response.

A Negative Feedback Loop in the Oxidative Stress Response—Even with continuous exposure to oxidative stress, the stabilization of RNT-1 proteins was abolished after 4 h (Fig. 2*B*). One possibility is that the transcriptional activation of *vhp-1* by RNT-1 could in turn diminish the stabilization of RNT-1 by dephosphorylating PMK-1, resulting in a decrease in the amount of phosphorylated RNT-1 proteins. To test this, we measured the extent of the stabilization of RNT-1 GFP proteins after 100 mM paraquat treatment in the *vhp-1* RNAi background (Fig. 6). In wild-type control animals, stabilized RNT-1 was increased by 30 min but abolished after 4-h treatment of 100 mM paraquat. In *vhp-1* RNAi animals, however, RNT-1 proteins were detected in the intestine even before paraquat treatment. Moreover, RNT-1 remained stabilized after 4-h exposure. These results suggest that stabilized RNT-1 is diminished by the decrease in active PMK-1, which is mediated by *vhp-1* transcription activated by RNT-1.

DISCUSSION

In this study, we revealed a novel function of RNT-1 in oxidative stress response in *C. elegans*. The data presented here show that ubiquitin-mediated degradation of RNT-1 is blocked by acute oxidative stress and that RNT-1 regulates stress response genes to increase viability. Stabilized RNT-1 activated the transcription of *vhp-1*, which, in turn, turns off the signal cascade of the p38 MAP kinase pathway. Overall, we propose a new role for *rnt-1* in the stress response with a regulatory feedback loop involving *vhp-1* (Fig. 7). Stabilized RNT-1 also activated stress response genes to cope with the stresses. The precise mechanism of the defensive role of RNT-1 target genes should be pursued further in the future. Further analysis of target genes in oxidative stress (supplemental Table 2) will extend the understanding of the role of RNT-1 in stress responses.

So far, studies of RNT-1 in the nematode have been focused on its role in development, such as proliferation and differentiation, and its transcriptional regulation. In this report, we, for the first time, showed that RNT-1 is controlled at the posttranslational level by environmental stress signals in the intestine. In *C. elegans*, one of the first organs that confront the changes of the environment is the intestine. Therefore, it is plausible that continuous expression and degradation of RNT-1 proteins in the intestine, not the regulation of *rnt-1* expression at the level of transcription, may be beneficial to the worms so that they can more rapidly respond to the environmental changes to protect them from damage. Degradation of RNT-1 proteins, seemingly in vain, may not be a waste of energy in nature because it is probable that the wild environment is not always as favorable to the worms as the laboratory culture condition and that RNT-1 may not be degraded all the time in the wild.

The MAP kinase pathways have been identified as the signaling pathways that transduce the environmental changes from

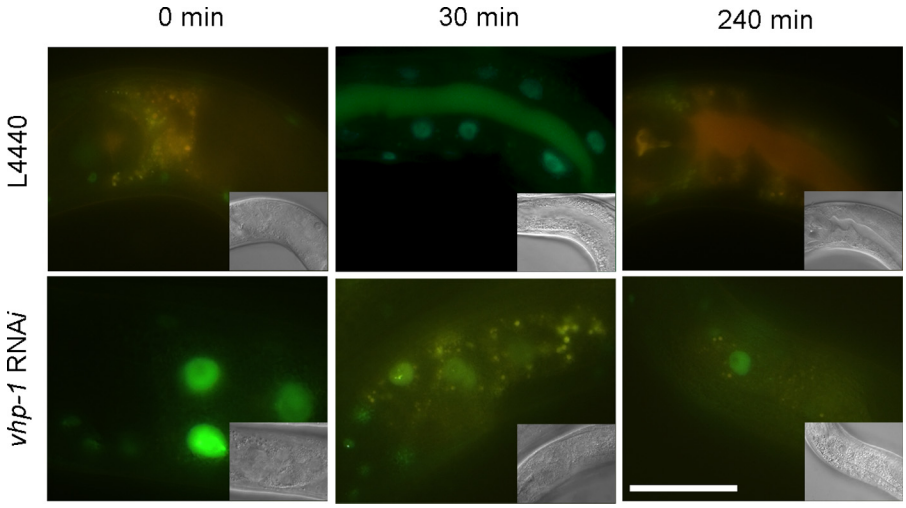

FIGURE 6. **The effect of the *vhp-1* RNAi on the stability of RNT-1 in the oxidative stress condition.** The photos show time course changes in the intestinal GFP of wild-type animals containing an RNT-1::GFP transgene in the control (*L4440*, *upper panels*) and *vhp-1* RNAi background (*bottom panels*). *Scale bar* = 25 μm.

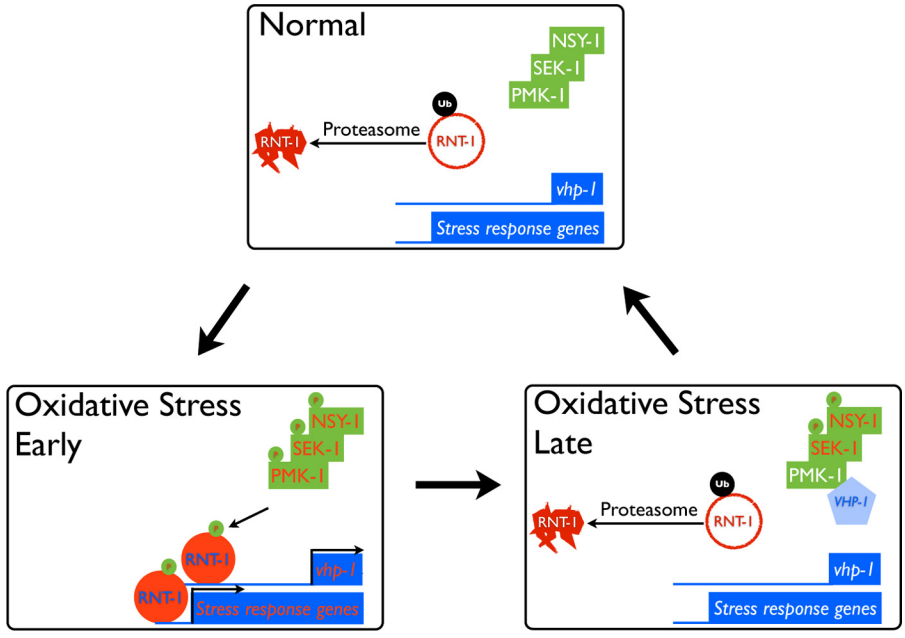

FIGURE 7. **A model of RNT-1 action in oxidative stress.** Oxidative stress induces the transcription of *vhp-1*, a phosphatase, which, in turn, negatively regulates the stabilization of RNT-1 in time under the oxidative stress condition.

outside of the cell or of an organism to the inside (38). In particular, the p38 MAP kinase has been known to have pivotal roles in stress response, immunological regulation, apoptosis, senescence, and cell cycle checkpoint. The p38 MAP kinase pathway in *C. elegans* also has well conserved functions in development, various stress responses, and innate immunity (39, 40). Phosphorylation of PMK-1, an ortholog of p38 MAP kinase, is increased by oxidative stress, pathogens, and osmotic shock (30, 33, 41). Among the downstream effector genes of the PMK-1 pathway there are several key transcription factors. One such protein is SKN-1, which is translocated from the cytosol to the nucleus upon oxidative stress (42). Recently, another transcription factor, ATF-7, was reported as a regulator of innate immunity controlled by the PMK-1 pathway (43). Our finding that oxidative stress induced the stabilization of RNT-1 protein in the nucleus through the PMK-1 pathway adds to the repertoires of the MAP kinase-regulated stress response mechanism.

It is not clear at this point how phosphorylation of RNT-1 proteins can protect them from ubiquitination-mediated proteasome action. However, protein stability augmentation by phosphorylation is not unprecedented. For example, c-Jun, a component of the AP-1 transcription factor complex, is an oncoprotein and is tightly regulated by its degradation mediated by three E3 ligases, COP1, Itch, and Fbw7 (44–46). c-Jun N-terminal Kinases (JNKs) are one of the kinases of c-Jun and protect c-Jun from degradation (47). The specific characteristics of E3 ligase for RNT-1 and its dynamic regulatory mechanisms upon oxidative stress still remain to be established. Identifying the E3 ligase of RNT-1 may contribute to the understanding of a main concept regarding the immediate

turnover mechanism of RUNX at the protein level. Excess responses and defects in the turning off of signaling events may cause diseases such as autoimmune disease and cancer development. The fact that RUNX family is involved in immune cell development and carcinoma in mammals encourages further study to discover a conserved feedback loop by posttranslational modification.

The MAP kinase phosphatase *vhp-1*, which detaches phosphorylation from MAP kinases, has been identified in other studies to regulate heavy metal stress and innate immunity in *C. elegans* (34, 35). We demonstrated the existence of the posttranslational modification mechanism for RNT-1 and a distinct type of regulatory feedback loop in the intestine of *C. elegans*.

Recently, there was a report that RUNX2 induces vesicular calcification in response to oxidative stress in mice (48), which supports the possibility of functional conservation in the oxidative stress response from nematodes to mammals. Others suggested that genetic alteration caused by impairment of the defense system against external chemicals or pathogenic bacteria, for example *Helicobacter pylori*, is the main cause of neoplastic transformation (49). In this context, RUNX3 might be required for various stress responses to protect the gastric epithelial cells from cellular transformation. At least in *C. elegans*, RNT-1 has a critical role in the stress response in the intestine, which is a primitive organ of mammalian gastrointestinal track systems. It would be interesting to examine the control of RUNX genes at the level of protein stability and their role in the stress response in addition to their developmental roles.

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