Studies on the Mechanisms Underlying Lifespan Regulation and Heat Stress Response of Transcription Factor DAF-16 in *Caenorhabditis elegans*

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Content

Chapter Ⅰ

Preface

Forkhead transcription factor DAF-16 is evolutionarily conserved from humans to worms (1). DAF-16 is localized downstream of the insulin-like signaling pathway and regulates the expression of genes for stress response, metabolism, signal transduction and longevity (2-4). It has been shown that DAF-16 and its orthologs respond to several environmental stimuli, such as heat, reactive oxygen species, and UV-irradiation, and are also involved in lifespan extension across species. In addition, recent studies have uncovered that the precise regulation of DAF-16 is attained by multiple posttranslational modifications (1, 5). However, its detailed mechanism remains elusive to date.

Genetic analysis in *C. elegans* has shown that *daf-16* is required for the long lifespan of *daf-2* mutants (6). Since the *daf-2* gene encodes an insulin-like receptor that functions upstream the PI3K-AKT signaling pathway and DAF-16 is repressed by AKT-mediated phosphorylation, DAF-16 activity is considered to contribute to longevity of *daf-2* mutants (7-9). However, it still remains unclear whether transcriptional activation of DAF-16 is necessary for lifespan extension. Therefore, in chapter Ⅱ, I attempted to generate a transcriptional-activity-deficient *daf-16ΔAD* mutant using the CRISPR/cas9 genome editing system, and investigated the role of transactivation function in long lifespan of *daf-2* mutants. Interestingly, although transcriptional activity of *daf-16ΔAD* mutant was almost abolished, the long lifespan of

daf-2 mutants was only moderately shortened when crossed with *daf-16ΔAD* mutants. Furthermore, the expression levels of several DAF-16 target genes related to longevity were comparable to that of long-lived *daf-2* mutant. These results demonstrate that transactivation function of DAF-16 is partially but not entirely responsible for lifespan extension of *daf-2* mutant.

In addition to lifespan extension, DAF-16 plays a key role in thermotolerance (10). Under heat stress conditions, DAF-16 is translocated into the nucleus and activates the transcription of target genes to prevent damage from noxious heat. Although there are several reports concerning signaling pathways involved in the thermal response of DAF-16, a thermo-sensor that transduces signals to DAF-16 in response to high temperature has not been identified. On the other hand, a recent study has shown that TRPA-1 acts as a cold-sensor in *C. elegans* and potentiates the transcriptional activity of DAF-16 (11). The *trpa-1* is a member of transient receptor potential (TRP) channel superfamily that is evolutionally conserved from worms to humans and senses various environmental stimuli. In chapter Ⅲ, I examined the role of TRP channels in the thermal response of DAF-16 and discovered that GTL-1 acts as a thermo-sensor of DAF-16 in *C. elegans*.

Posttranslational modification is important for precise regulation of transcriptional activity of DAF-16 (1, 5). We previously reported that the protein arginine methyltransferase PRMT-1 directly methylates DAF-16 and thereby extends lifespan in *C. elegans* (12). PRMT-1 is a major enzyme for catalyzing asymmetric arginine dimethylation of cellular proteins and contributes to various biological processes (13,

14). Nevertheless, the mechanism underlying the regulation of PRMT-1 activity remains largely unknown. In chapter Ⅳ, I explored novel regulators of *prmt-1* expression using unbiased RNAi screen in *C. elegans*. I found that a GATA transcription factor ELT-2 regulates the mRNA and protein levels of PRMT-1, while ELT-2 interferes with the methyltransferase activity of PRMT-1, probably through direct interaction. Taken together, these results illustrate the two modes of PRMT-1 regulation, which could determine the levels of asymmetric arginine dimethylation in *C. elegans*.

Chapter Ⅱ

DAF-16 transactivation deficient mutant partially reduced lifespan extension of *daf-2* long-lived mutant

Summary

Forkhead transcription factor DAF-16 regulates genes for stress response, metabolism and longevity in *C. elegans*. Genetic analysis has shown that *daf-16* is required for the long lifespan of *daf-2* mutants. Since the *daf-2* gene encodes an insulin-like receptor that functions upstream the PI3K-AKT signaling pathway and DAF-16 is repressed by AKT-mediated phosphorylation, DAF-16 activity is considered to contribute to longevity of *daf-2* mutants. However, it still remains unclear whether transcriptional activity of DAF-16 is necessary for lifespan extension.

To address this issue, we attempted to generate loss of function mutants of *daf-16* using CRISPR/cas9 system. We have established *daf-16 ΔAD* mutant that harbors mutated *daf-16* allele lacking 26 amino acidss of the C-terminal transactivation domain. I first confirmed that transactivation function of DAF-16 ΔAD is largely abolished using *in vivo* worm luciferase assay. Moreover, biochemical analysis showed that *DAF-16 ΔAD* mutant fail to interact with the transcriptional co-activator CBP, while it is able to bind to the consensus DNA sequence of DAF-16. Surprisingly, *daf-16 ΔAD* mutant exhibited only partially reduced lifespan extension of *daf-2* mutant. Supporting

these results, mRNA-seq analysis revealed that *daf-2;daf-16 ΔAD* mutant expressed ubiquitin-dependent protein catabolic process genes, which are regulated by DAF-16 and contribute to longevity, to a similar extent in *daf-2* mutant. Taken together, these results demonstrated that transactivation of DAF-16 is largely, but not entirely, responsible for long lifespan of *daf-2* mutant, and non-transcription function of DAF-16 may contributes to lifespan extension.

Chapter Ⅲ

TRP channel GTL-1 function as a thermo-sensor of DAF-16 heat stress response

Summary

DAF-16 plays a key role in heat stress resistance, in similar to lifespan extension, in *C. elegans*. Under heat stress condition, DAF-16 translocates to the nucleus and enhances the transcription of target genes to prevent damage from noxious heat. Previous studies found various factors involving in the regulation of DAF-16 thermal response, for example, *jnk-1*, *unc-43* and *tax-6*. Nevertheless, a thermo-sensor that transduces signal to DAF-16 in response to temperature elevation has not been discovered yet.

On the other hand, a recent study has shown that TRPA-1 acts as a cold-sensor in *C. elegans* and potentiates the transcriptional activity of DAF-16. The *trpa-1* is a member of transient receptor potential (TRP) channel superfamily, which is highly conserved from humans to worms. The TRP family is known to be a cation channel to maintain intracellular homeostasis and paly important roles in sensation of environmental stimuli.

Thus, I explored whether TRP channel function as a thermo-sensor of DAF-16 heat stress response using *in vivo* worm-luc system. As a result of an RNAi screening of

TRP channel genes, I found that *gtl-1* RNAi, which is an ortholog of mammalian *trpm3*, decreased luciferase activity under heat stress condition. *gtl-1* null mutant also reduced luciferase activity under heat stress condition, but showed marked increased of luciferase activity same extend as wild-type under starve condition. In addition, *gtl-1* RNAi mitigated heat-induced nuclear accumulation of DAF-16. The *gtl-1* mutant exhibited a reduced heat stress resistance, while there were no effect on resistance to oxidative and UV-irradiation stresses. Furthermore, I attempted to define the epistatic relationship between *daf-16* and *gtl-1*, and reveled that heat stress resistance of *gtl-1;daf-16* double mutant is comparable to that of *daf-16* mutant. This result demonstrated that DAF-16 is regulated downstream of GTL-1. Taken together, my study provided evidence that TRP channel, GTL-1, is a thermo-sensor of DAF-16 in *C. elegans*.

Chapter Ⅳ

The GATA transcription factor ELT-2 modulates both the expression and methyltransferase activity of PRMT-1 in *Caenorhabditis elegans*

Summary

Protein Arginine Methyltransferase 1 (PRMT1) catalyzes asymmetric arginine dimethylation of cellular proteins and thus modulates various biological processes, including gene regulation, RNA metabolism, cell signaling and DNA repair. Since loss-of function mutation of *prmt-1* completely abolishes asymmetric dimethylarginine in *C. elegans*, PRMT-1 is thought to play a crucial role in determining levels of asymmetric arginine dimethylation. However, the mechanism underlying the regulation of PRMT-1 activity remains largely unknown. Here I explored for transcription factors that induce the expression of PRMT-1 by an RNAi screen using transgenic *C. elegans* harboring *prmt-1* promoter upstream of *gfp*. Of 529 clones, I identify a GATA transcription factor *elt-2* as a positive regulator of *Pprmt-1::gfp* expression and show that *elt-2* RNAi decreases endogenous PRMT-1 expression at mRNA and protein levels. Nevertheless, surprisingly arginine methylation levels are increased when *elt-2* is silenced, implying that ELT-2 may also have ability to inhibit methyltransferase activity of PRMT-1. Supporting this idea, GST pull-down and co-immunoprecipitation assays demonstrate the interaction between ELT-2 and PRMT-1. Furthermore, I find that

ELT-2 interferes with PRMT-1-induced arginine methylation in a dose-dependent manner. Collectively, my results illustrate the two modes of PRMT-1 regulation, which could determine the levels of asymmetric arginine dimethylation in *C. elegans*.

Introduction

Among post-translational modifications, protein arginine methylation is as common as phosphorylation and ubiquitination, and now attracting increasing attention because of its various roles in epigenetic regulation, pre-mRNA splicing, mRNA translation, cell signaling, DNA damage response, and cell fate decision (13, 14, 15). Arginine methylation is classified according to the number and position of the methyl groups on a guanidino nitrogen atom of arginine residue: w-N^G-monometylarginine (MMA), w-N^G, N^G-asymmetric dimethylarginine (ADMA), and w-N^G, N'^G-symmetric dimethylarginine (SDMA), and protein arginine methyltransferases (PRMTs) are responsible for catalyzing the transfer of a methyl group from S-adenosylmethionine (SAM) to a target arginine residues (14, 16). In mammals, members of PRMT family fall into three groups based on the end products, namely type I (PRMT1, PRMT2, PRMT3, PRMT4, PRMT6, PRMT8, PRMT10 and PRMT11) and type II (PRMT5 and PRMT9) enzymes carry out the formation of MMA as an intermediate, and then type I and II further catalyze the production of ADMA and SDMA, respectively (17). Another is a type III enzyme consisting of PRMT7 alone that catalyzes only the formation of MMA (18). Above all, PRMT1 is known to be the predominant type I enzyme in mammalian cells (19, 20) and essential for embryonic development in mice and *Drosophila* (21, 22). In contrast, our laboratory have previously reported that the null mutant of nematode *Caenorhabditis elegans* PRMT-1 is viable, albeit complete loss of

ADMA (12, 23), which in turn prompted us to use *C. elegans* as a model for examining the regulatory mechanism of PRMT-1 *in vivo*.

Cellular arginine methylation levels are considered to be determined by PRMT activity, since the existence of arginine demethylases is controversial to date (17, 24). In the case of PRMT1, its activity is differentially regulated by two distinct modes: first is at the transcriptional level and second is at the post-transcriptional level. For example, a recent study has reported that the expression of PRMT1 is induced by the IL-4/STAT6 signaling pathway in epithelial cells, while in fibroblasts PRMT1 is upregulated by IL-1β through NF-κ B (25). On the other hand, PRMT1 was identified as a binding partner for the antiproliferative protein BTG1 and the related TIS1 proteins, and their interaction resulted in enhancing the methyltransferase activity of PRMT1 towards selected substrates *in vitro* (26). In addition, it was reported that BTG1-binding protein hCAF1 also binds to PRMT1 and modulates the methylation of endogenous substrates, such as Sam68 and histone H4, in MCF-7 cells (27). However, these findings were obtained from the examination using yeast or cultured cells, therefore; the mechanism regulating PRMT1 activity in a multicellular organism remains still unknown.

In this study, I searched for novel transcription factors that upregulates the expression of PRMT1 by using a transgenic *C. elegans* harboring a *prmt-1* promoter-fused GFP gene. An RNAi screening identified a GATA transcription factor *elt-2* as key modulator of *prmt-1* gene expression and I illustrated that *elt-2* is indeed involved in a basal expression of PRMT-1. Surprisingly, however, *elt-2* RNAi increased asymmetric arginine dimethylation on cellular proteins in *C. elegans*. Given these

controversial findings, I hypothesized that while ELT-2 induces PRMT-1 expression as a transcription factor, it also might inhibit an enzymatic activity of PRMT-1, likely through protein-protein interaction. Supporting this hypothesis, I demonstrated that ELT-2 binds to PRMT-1 by GST pull-down and co-immunoprecipitation assays. Furthermore, I provided evidence that ELT-2 interferes with methyltransferase activity of PRMT-1 *in vitro*. Taken together, my findings identify the two modes of PRMT-1 regulation as a potential determinant of cellular asymmetric arginine dimethylation levels in *C. elegans*.

Materials and methods

C. elegans Strains and Maintenance

Bristol N2 wild-type and RB2047:*prmt-1(ok2710) V* were obtained from the Caenorhabditis Genetics Center (University of Minnesota, USA). Worms were maintained on NGM plates seeded with a lawn of the *E. coli* strain OP50 at 20°C and handled according to standard methods .

Generation of Transgenic Line

Transgenic strains carrying extrachromosomal arrays were generated using standard microinjection methods. For generating TKB311: *trcIs34[Pprmt-1::gfp]* transgenic animals, a *Pprmt-1::gfp* plasmid containing the promoter region (3164 bp) of the *prmt-1* gene was ligated to the pPD95_81 vector and then injected at 100 ng/μl together with *myo-2::DsRed* marker plasmid at 5 ng/μl into N2 wild-type. The extrachromosomal array was integrated by UV irradiation and then backcrossed to N2 three times.

RNAi screen

RNAi knockdown by feeding was performed essentially as described previously (29). As an RNAi library of transcription factors in *C. elegans*, we prepared the predicted 529 genes consisting of originally constructed and the Ahringer library clones. For RNA screen, the frozen stocks of HT115 bacteria carrying RNAi clones or L4440

empty vector as control were used to inoculate 800 μl LB supplemented with 50 μg/ml ampicillin. After an overnight culture, IPTG was added to 1 mM and further grown for 2 h. NGM plates containing 5 mM IPTG were seeded with 30 μl of bacteria and allowed to dry. Synchronized L1 larvae were used to seed prepared RNAi plates. Following 48 h culture, animals were visually examined for changes in GFP fluorescent.

Feeding RNAi

The RNAi clone against *elt-2* was obtained from the Ahringer library. After confirming the sequence, the *elt-2* RNAi construct was transformed into HT115 and then inoculated in 2 mL LB supplemented with 50 mg/mL ampicillin for overnight. Bacteria were pelleted and resuspended in 10% of the original volume. NGM plates containing 25 mg/ml carbenicillin and 1 mM IPTG were seeded with concentrated bacteria and allowed to dry. Synchronized L1 larvae were used to seed prepared RNAi plates.

GFP Fluorescent Observation and Quantification

For measuring the intensity of GFP, worms were mounted on glass slides in 100 mM sodium azide and visualized using the IX73 inverted microscope system (OLYMPUS). Fluorescent images were taken using the Microsystem Frame Work software with a DFC500 camera (Leica). After conversion from color to grey-scale, the fluorescence in whole body was quantified by measuring the pixel intensity using

Image-Pro Plus Application (Leica). At least three worms in each clone were measured and mean values were calculated.

Quantitative RT-PCR

N2 animals were grown on RNAi plates at 20 °C until the young adult stage and then total RNAs were extracted with ISOGEN II (Nippon Gene). Five hundred ng of total RNA were reverse transcribed using ReverTra Ace (Toyobo) and then real-time quantitative PCR reactions were performed using a SYBR Premix ExTaq II (Takara). Relative gene expression was determined by *ΔΔ*Ct method, normalized to *act-1* level. The following primers were used.

prmt-1: Forward 5'-AGACGCGCTCACAGTAAAGAAAG-3'

prmt-1: Reverse 5'-CAACGAGCAGAACACCTACACC-3'

act-1: Forward 5'-CAACGAGCAGAACACCTACACC-3'

act-1: Reverse5'-CTTCTGTCCCATACCGACCA-3'

Cell culture and plasmid transfection

HEK293T cells were cultured in DMEM supplemented with 10% FBS. Plasmid transfection was performed using GeneJuice Transfection Reagent (Merck) according to the manufacture's protocol.

Western Blot and Co-immunoprecipitaion

N2 animals were grown on RNAi plates at 20 °C until the young adult stage and

washed off the plates with M9 buffer. The worms were sonicated in the worm lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% NP-40 and protease inhibitors) and then centrifuged to remove insoluble material. The supernatants were analyzed by Western blot with anti-PRMT-1 (12), anti-αTubulin (Sigma), ASYM24 (Merck), and ASYM26 (Epicypher) antibodies. For co-immunoprecipitation assays, HEK293T cells transfected with both pcDNA3-FLAG-ELT-2 and pcDNA3-HA-PRMT-1 were lysed with the cell lysis buffer. After centrifugation, the whole-cell lysates were immunoprecipitated using anti-FLAG M2 affinity gel (Sigma), followed by western blotting with anti-FLAG (M2, Sigma) and anti-HA (3F10, Sigma) antibodies.

GST pull-down assay

GST-fused PRMT-1 proteins expressed in *E. coli* strain BL-21 by using the pGEX vector system were immobilized on glutathione-Sepharose and incubated with cell extracts from HEK293T cells expressing FLAG-ELT-2 proteins. The component of cell lysis buffer was as follows: 20 mM HEPES (pH7.5), 150 mM NaCl, 0.1% TritonX-100 and protease inhibitors. After incubation for 4 h at 4 °C, the beads were washed three times with the same buffer, and proteins were analyzed by Western blotting with an anti-FLAG antibody (MBL).

Purification of FLAG-ELT-2 proteins

FLAG-ELT-2 proteins expressed in HEK293T cells were immunoprecipitated with

anti-FLAG M2 affinity gel. The beads were suspended in TBS buffer containing 3X FLAG peptide (150 ng/μl, Sigma) in a final volume of 100 μl with gentle shaking at 4 °C for 30 min. After quick centrifugation, the supernatants were transferred to fresh tubes and used as FLAG-ELT-2 protein solution.

In vitro Methylation assay

In vitro methylation assays were performed as described previously with some modification (30). Briefly, four μg of GST-RGG3 (543-656 aa of human EWS) proteins were preincubated with increasing amount (0, 20, 40, 80 μl) of eluted FLAG-ELT-2 proteins in a final volume of 100 μl of TBS buffer on ice for 1 h. Thereafter, two μg of GST-PRMT-1 and 1 μl of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (37 KBq/nmol) were added to the reactions and further incubated at 25 °C for 1 h. After washing the beads, the reaction products were analyzed by CBB staining, followed by treatment with Amplify Fluorographic Reagent (GE Healthcare). The gel was dried and then exposed to high performance autoradiography film (GE Healthcare) at -80 ˚C for 3 days.

Acid Hydrolysis and LC-MS/MS

Acid hydrolysis of worm proteins was performed as previously described (12). Briefly, fifty microgram of worm proteins were hydrolyzed with 6 N HCl at 110 °C for 24 h in glass vials. *N*-propyl-L-arginine (N-PLA) was added as the internal control. ADMA and MMA were quantified using a NexeraTM ultra high-pressure liquid chromatography system coupled to LCMS-8050TM triple quadrupole mass

spectrometer (Shimadzu) as described previously (31). All analyses and data processing were completed with the LabSolutions Ver. 5.60 software (Shimadzu).

Statistical Analysis

Results were presented as mean ± standard deviation (SD). Statistical significances were determined by two-tailed unpaired Student's t-test using GraphPad Prism 6 (GraphPad software). Significant differences are indicated as follows: **$p<0.001$, ***$p<0.0001$.

Result

An RNAi Screen of Transcription Factors Using *Pprmt-1::gfp* Transgenic Worms

In order to explore for trans-regulator(s) of *prmt-1* gene expression *in vivo*, I first generated a transgenic nematode strain stably harboring a *gfp* reporter that is driven by the promoter region of *prmt-1* upstream from the start codon. Consistent with previous reports (12), the *Pprmt-1::gfp* transgenic strain exhibited broad expression patterns of GFP, including head neuron, pharynx, intestine, and body wall muscle (data not shown). Additionally, I decided to measure the intensity of fluorescence at the young adult stage when *Pprmt-1::gfp* expression reaches a peak throughout life. For unbiased genome-wide screen, I prepared an RNAi feeding library containing the predicted 528 clones of transcription factors in *C. elegans* and the transgenic worms synchronized at the L1 larval stage were grown on RNAi plates for 48 hrs, followed by visualization and quantification of GFP.

The ELT-2 Transcription Factor Was Identified as a Regulator of *prmt-1* Gene Expression

Since I searched for trans-activators of prmt-1, GFP fluorescence should become darker compared to control. Our RNAi screen identified twelve genes that resulted in more than 30% reduction of GFP intensity when silenced from hatching. In particular, among these candidates, I focused on the most effective one: *elt-2*, which encodes a GATA transcription factor homologous to human GATA4. *elt-2* is known to be

expressed solely in the intestine and play a essential role in inducing a number of intestine-specific terminal differentiation genes as well as genes involved in the intestinal innate immune responses to bacterial and fungal infection (31-33). Indeed, we found that *elt-2* RNAi treatment reduces *Pprmt-1::gfp* expression exclusively in the intestine by as much as 50%. These results indicate that ELT-2 is a positive regulator for expression of the *prmt-1* gene *in vivo*.

elt-2 Is Required for an Endogenous Expression of *prmt-1*

I next investigated whether *elt-2* regulates endogenous expression of the *prmt-1* gene in *C. elegans*. Wild-type N2 animals were fed on *elt-2* RNAi plates from hatching and then the amounts of PRMT-1 protein were detected using a *C. elegans* PRMT-1-specific antibody. As expected, depletion of *elt-2* resulted in a decrease in PRMT-1 protein compared with control knockdown. Furthermore, I demonstrated that *elt-2* RNAi significantly decreases mRNA levels of *prmt-1*.

Given that the intestinal expression is only a portion of total *prmt-1* (12), these data suggest a large contribution of ELT-2 to the *prmt-1* gene expression at the level of transcription.

elt-2 RNAi Increases Asymmetric Arginine Dimethylation on Cellular Proteins in *C. elegans*

Based on our findings, it is expected that *elt-2* RNAi could decrease the levels of

asymmetric arginine dimethylation as a consequence of a decline in PRMT-1. To test this assumption, whole cell lysates from control and *elt-2* knockdown animals were analyzed by Western blotting with two distinct anti-asymmetric-dimethylarginine antibodies, ASYM24 and ASYM26 (33). Contrary to expectations, however, *elt-2* RNAi led to a marked increase in levels of ADMA on several cellular proteins ranging from 17 to 75 kDa when compared with controls. These unexpected results prompted to verify the *elt-2* RNAi-induced elevation of ADMA levels by using a liquid chromatography system coupled to triple quadrupole mass spectrometer (LC-MS/MS). Total proteins from control or *elt-2* RNAi animals were acid hydrolyzed to free amino acids, and the products were separated and quantified with LC-MS/MS. Cellular ADMA levels of *elt-2* RNAi animals were 2.4-fold higher than controls. Interestingly, it should be noted that monomethyl arginine (MMA) was also increased to a greater extent (5.7-fold) by silencing *elt-2*, probably as an accumulation of intermediate for catalyzing the formation of ADMA. To further confirm that the accumulation of ADMA and MMA in *elt-2* knockdown animals is dependent on the methyltransferase activity of PRMT-1, we conducted *elt-2* RNAi on the *prmt-1(ok2710)* null mutant. As

expected, *elt-2* RNAi had no effect on both the ADMA and MMA levels in the *prmt-1(ok2710)* null mutant. Taken together, these results indicate that *elt-2* RNAi-induced reduction in PRMT-1 expression involves the simultaneous activation of PRMT-1 activity in *C. elegans*.

ELT-2 Binds to PRMT-1 *in Vitro* and *in Cells*

One possible explanation for this unexpected finding was that ELT-2 protein might inhibit an enzymatic activity of PRMT-1, likely through protein-protein interaction. To test this possibility, I first performed GST pull-down assays using *C. elegans* PRMT-1 and ELT-2 proteins expressed in *E. coli* and mammalian cells, respectively. As expected, I found that FLAG-tagged ELT-2 binds to GST-fused PRMT-1 but not GST alone *in vitro*. Next, I asked if this interaction also occurs *in vivo* by transfecting mammalian cells with expression plasmids of both ELT-2 and PRMT-1. Western blot analysis of anti-FLAG immunoprecipitates showed that ELT-2 binds specifically to PRMT-1 when ectopically overexpressed in HEK293T cells. Collectively, these results suggest that ELT-2 is a binding partner of PRMT-1 *in vitro* and *in cells*.

ELT-2 Inhibits Enzymatic Activity of PRMT-1 *in Vitro*

Since *elt-2* RNAi experiments did not allow us to propose a mechanism whereby reduction of ELT-2 increases cellular arginine methylation in *C. elegans*, we conducted

an *in vitro* methylation assay to address this question. To this end, I utilized a recombinant fragment of human EWS protein harboring arginine- and glycine-rich motif, termed RGG box, that is the most common sequence preferred by PRMT1 (35). In addition, as a possible inhibitor of methylation reaction, FLAG-ELT-2 proteins expressed in HEK293T cells were purified by immunoprecipitation and subsequent elution with the epitope peptide, and then added to the reaction solution containing PRMT-1 and RGG with serial amounts. PRMT-1-induced methylation was readily detected in GST-fused RGG proteins, but not GST alone. Importantly, addition of ELT-2 decreased methylation levels of RGG, and this reduction in the RGG methylation was inversely correlated with increased amount of ELT-2 protein. Considering this result together with a possible interaction between ELT-2 and PRMT-1, the increase in ADMA levels by *elt-2* RNAi appears to be due to a depletion of ELT-2 that could be an inhibitor of PRMT-1 activity through direct binding.

Discussion

In this report, we identified ELT-2 as a transactivator of the *prmt-1* gene by an unbiased RNAi screen using the *Pprmt-1::gfp* transgenic *C. elegans*. It has been reported that the majority of genes expressed exclusively or primarily in *C. elegans* intestine include a TGATAA-like sequence served as a strong ELT-2 binding site in their promoters (*33, 36*). Unexpectedly, however, that sequence was not found in the *prmt-1* promoter. Given the ubiquitous expression pattern of *prmt-1* (*12*), it seems likely that ELT-2 may recognize other *cis*-acting elements in the *prmt-1* promoter. Alternatively, our screening method does not exclude the possibility that ELT-2 may function as a transcriptional coactivator that modulates the *prmt-1* gene expression. Thus, we argue here that ELT-2 plays a positive role in intestinal expression of PRMT-1 in *C. elegans*.

Besides its role in the intestine, our data that *elt-2* RNAi decreases the *Pprmt-1::gfp* expression in neurons and muscles imply non-cell autonomous effects of ELT-2 on PRMT-1 expression. How does ELT-2 control the expression of *prmt-1* in remote tissues? Considering that ELT-2 is known to be required for developing a normal intestinal structure (*32*), a plausible hypothesis is that *elt-2* RNAi-induced intestinal dysfunction decreases nutrient intake and thereby causes a perturbation of endocrine signals from the intestinal cells, which in turn might downregulate the expression of PRMT-1 in distal tissues. If so, a reduction of PRMT-1 except in the intestine could be due to an indirect and secondary effect of *elt-2* knockdown.

In addition to ELT-2-dependent *prmt-1* expression, I had an unexpected finding that *elt-2* RNAi increases the levels of ADMA in *C. elegans*. It appears to be controversial because if ELT-2 acts as a transactivator of *prmt-1*, *elt-2* RNAi could lead to a reduction of PRMT-1 and thereby results in an decrease in ADMA levels. Yet, I was able to obtain reasonable findings in this context, namely ELT-2-dependent inhibition of PRMT-1 activity by *in vitro* methylation assay. I also demonstrated a direct interaction between ELT-2 and PRMT-1. These findings led me to propose a simple model in which ELT-2 binds competitively to PRMT-1 and interferes with the proper interaction between PRMT-1 and its substrate proteins, which in turn, inhibits asymmetric arginine dimethylation of target sites. Further investigations to identify PRMT-1 domain(s) where ELT-2 interacts are needed to elucidate a detailed mode of inhibitory action of ELT-2 on methyltransferase activity of PRMT-1.

Here I describe a novel two-modes of PRMT-1 regulation by ELT-2 as described above. It is an important question how such “negative feed-back” mechanism contributes to biological significance of PRMT-1. I interpret the present results as supporting the hypothesis that ELT-2 promotes asymmetric arginine dimethylation when it acts as a transactivator by upregulating the *prmt-1* expression, while once ELT-2 dissociates from DNA, the “free” ELT-2 reduces the methylation levels through competitive antagonism of PRMT-1. Based on my present data that *elt-2* RNAi from the L1 larvae to the young adult stages increases ADMA levels, the proportion of DNA-unbound ELT-2 is assumed to be relatively high during larval development. In contrast, throughout embryogenesis from the early 2E-cell stage, ELT-2 acts as the

master regulator directly controlling many “effector” genes required for intestinal development (31, 32, 36), ELT-2 functions on PRMT-1 activity may be shifted primarily to the *prmt-1* expression as well as other target genes.

Our laboratory previously reported that PRMT-1 contributes to lifespan extension by activating the forkhead box O (FOXO) transcription factor, DAF-16 (12). DAF-16 is a primary target of insulin/IGF-1 signaling, and multiple isoforms of DAF-16 (a, b, and d/f) are known to play central roles in various biological processes, including lifespan, fat storage, and stress resistance (37). Interestingly, a recent study shown that the intestinal expression of *daf-16d/f*, an isoform responsible for longevity, is upregulated by ELT-2 at the level of transcription (38). These results, combined with the present study, raise the possibility that *elt-2* could be an upstream determinant of longevity by controlling the expression of both *prmt-1* and *daf-16d/f* in the intestine. More recently, in support of this hypothesis, Mann, *et al*. identified ELT-2 as a direct regulator of aging transcriptome by screening with the modENCODE ChIP-seq data (39). They found that *elt-2* expression begins to decline with age, whereas overexpression of *elt-2* extends lifespan and slows the rate of gene expression changes that occur during normal aging (39). Thus, in view of the role of ELT-2 in adulthood, it seems likely to be an intestinal master regulator that determines longevity by altering a network of transcription in *C. elegans*.

Finally, My present findings provide new insight into the regulatory mechanism of PRMT-1 by utilizing the *C. elegans* model. Unlike other organisms, the null mutants of *C. elegans prmt-1* and *prmt-5* are both viable with no severe phenotype, despite loss of

asymmetric and symmetric arginine dimethylation, respectively (40). Considering the conservation of PRMT proteins and their cellular functions from worm to mammals, *C. elegans* could be valuable model system for investigating the regulatory mechanism of PRMT family members *in vivo*.

Chapter Ⅴ

Concluding Remarks

Accumulating evidence suggested that transcription factor DAF-16 plays a critical role in multiple biological processes, such as stress response, metabolism and lifespan extension across species. For now, many groups around the world have revealed numerous regulation mechanisms of DAF-16. Nevertheless, more its detailed mechanisms have not been elucidated.

In chapter Ⅱ, since transcriptional activity of DAF-16 is considered to contribute to lifespan extension, I investigated to whether transactivation function of DAF-16 is necessary for lifespan extension. I demonstrated that DAF-16 dependent transcription is largely, but not entirely, responsible for lifespan extension. Interestingly, it appears that transcriptional activity independent function of DAF-16 is also required for lifespan extension. Supporting this idea, it is reported that DAF-16 acts in several biological processes in transcription independent manner. In this study, transactivation deficient DAF-16 mutant showed DNA binding ability as a same extent to DAF-16 wild-type. This finding provides the possibility that not only transcriptional activity but also DNA binding mediated transactivation independent functions are important for DAF-16 induced lifespan extension.

In chapter Ⅲ, to reveal the mechanism of DAF-16 heat stress response, I explored a thermo-sensor of DAF-16. I found that TRP channel, GTL-1, is required for DAF-16 thermal response and DAF-16 is regulated downstream of GTL-1. My study provides

evidence that GTL-1 as a thermo-sensor of DAF-16 in *C. elegans*. Since, in this study, I did not explain how GTL-1 transduces the signal to DAF-16, further research is needed to elucidate the signal cascade. TRP channel genes are conserved in human, it is expected that FOXO, the mammalian ortholog of DAF-16, is also regulated by GTL-1.

In Chapter Ⅲ, I discovered a GATA transcription factor ELT-2 regulates the expression level of PRMT-1, which is modulator of DAF-16 transcriptional activity, on the other hand, ELT-2 directly binds to PRMT-1 and inhibits methyltransferase acitivity of PRMT-1. Taken together, these results illustrate the two modes of PRMT-1 regulation, which could determine the levels of asymmetric arginine dimethylation in *C. elegans*.

In conclusion, DAF-16 is important factor for lifespan extension and heat stress resistance, but its detailed mechanisms remain elusive to date. My novel finding may support the detailed understanding of lifespan regulation and thermal response of DAF-16.

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