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FEBS Letters 584 (2010) 3609–3614

FEBS Letters
journal homepage: www.FEBSLetters.org

# The *Drosophila* homolog of methionine sulfoxide reductase A extends lifespan and increases nuclear localization of FOXO

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ARTICLE INFO

*Article history:*
Received 30 March 2010
Revised 28 June 2010
Accepted 19 July 2010
Available online 23 July 2010

Edited by Vladimir Skulachev

*Keywords:*
Antioxidant enzyme
Drosophila
Forkhead box O
Lifespan
Longevity
Methionine sulfoxide reductase A
Oxidative stress
Transcription
Translocation

ABSTRACT

**Methionine sulfoxide reductase A (msrA) was previously found to increase resistance to oxidative stress and longevity in animals. We identified *Drosophila* msrA (*dmsrA*), a *Drosophila* homolog of human *msrA*, as a downstream effector of forkhead box O (FOXO) signaling in *Drosophila*, which enhances resistance to oxidative stress and increases survival under stressed conditions. Additionally, overexpression of *dmsrA* in neurons extended the lifespan of flies. Moreover, overexpression of *dmsrA* in fat body cells caused FOXO to translocate to the nucleus, implying that this possible positive feedback loop between *dmsrA* and FOXO could potentiate the antioxidant activity of *dmsrA* and increase the lifespan in *Drosophila*.**
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## 1. Introduction

Methionine sulfoxide reductases (msrs), which are found in most organisms from bacteria to humans, are thioredoxin-dependent repair enzymes that reduce methionine sulfoxide to form methionine [1–3]. Methionine sulfoxide reductase A (msrA) and msrB are specific for the S- and R-forms of methionine sulfoxide, respectively. Msrs are unique antioxidant enzymes that can repair oxidative damage to proteins [4–6]. The accumulation of oxidative damage, particularly to proteins, has been suggested as an important determinant of mammalian lifespan [4,7]. The important biological functions of msrA are well documented. Lack of msrA in *Escherichia coli* and yeast causes increased sensitivity to oxidative stress [8,9]. MsrA knockout mice display increased susceptibility to oxidative damage that results in either a 40% reduction in lifespan and neurological abnormalities [7] or in an undiminished lifespan accompanied by maintained neuromuscular function [10]. By contrast, overexpression of msrA in yeast and mammalian cell lines increases their resistance to oxidative stress [11–13]. Additionally overexpression of bovine msrA in *Drosophila* neurons resulted in increased lifespan, fertility, and resistance to paraquat-induced oxidative stress [14].

The mechanisms of *Drosophila msrA* (*dmsrA*) regulation and the identity of downstream signals that confer protection against oxidative stress and aging remain unknown. By reducing methionine sulfoxide groups on many enzymes and proteins to methionine, msrA may serve as a catalytic antioxidant system without any specific direct repair targets [15]. However, several targets of msrA

*Abbreviations:* JNK, c-Jun N-terminal kinase; dFOXO, *Drosophila* FOXO; dmsrA, *Drosophila* msrA; FOXO, forkhead box O; msrA, methionine sulfoxide reductase A; Prx II, Peroxiredoxin II; UAS, upstream activator sequence; GMR, glass multiple reporter

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doi:10.1016/j.febslet.2010.07.033

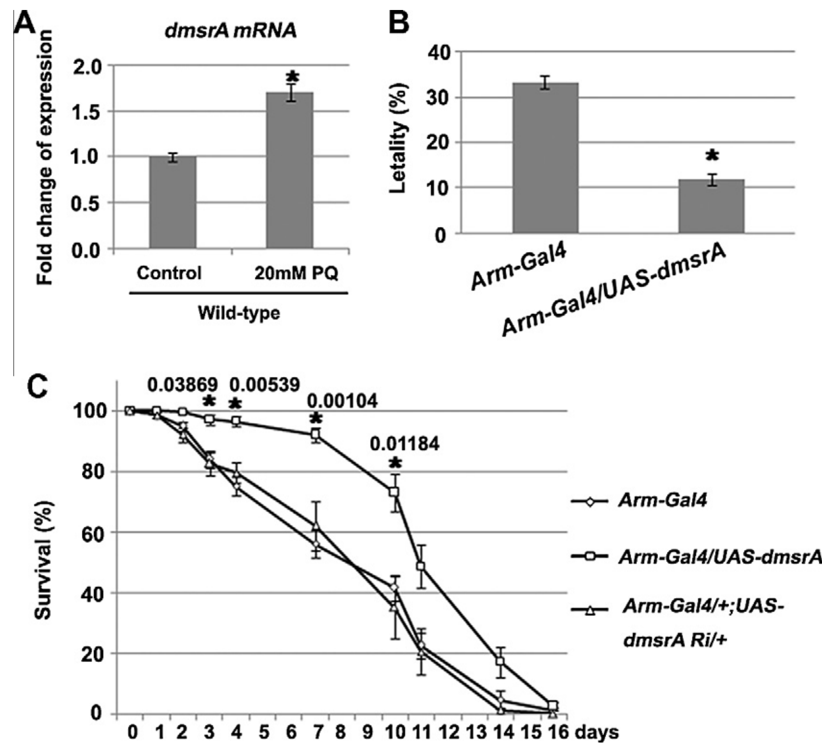



**Fig. 1.** Overexpression of *Drosophila msrA* (*dmsrA*) suppresses oxidative stress-induced lethality. (A) When oxidative stress was induced using 20 mM paraquat (PQ) in wild-type *Drosophila melanogaster*, the expression level of *dmsrA* increased compared to the control in the real-time RT-PCR analysis. Overexpression of *dmsrA* in the whole body (*Arm-Gal4/UAS-dmsrA*) suppressed the oxidative stress-induced lethality at 24 h (B) and increased the survival rate (C) compared to the *Arm-Gal4* control. However, the survival rate of *dmsrA* inhibition (*Arm-Gal4/+;UAS-dmsrA Ri/+*) was similar to that of the control (C). Data is represented as mean ± S.E. from three independent experiments ($n = 50$ for B; $^*P < 0.05$, Student's *t*-test).

have been identified, including the small heat shock protein, Hsp-21 [4], ribosomal protein L12 [16], calmodulin [17], and α-synuclein [18]. Recently, Minniti et al. [19] demonstrated that *msrA-1* might be regulated by DAF-16/FOXO3a, a conserved protein that regulates longevity in *Caenorhabditis elegans*. This finding suggested a possible regulatory mechanism for *msrA-1* that links its biochemical activity of repairing oxidative damage with its role in organism's lifespan.

In *Drosophila*, neuronal activation of c-Jun N-terminal kinase (JNK)/forkhead box O (FOXO) signaling is known to confer resistance to oxidative stress and extend lifespan [20,21]. FOXO transcription factors are key regulators of growth, metabolism, lifespan, and stress resistance in various organisms, including *Drosophila* [22,23]. FOXO is regulated by the insulin signaling pathway and the stress-induced JNK signaling pathway [21,24]. Oxidative stress activates JNK, which promotes FOXO nuclear localization, resulting in increased expression of antioxidant proteins [25,26].

We recently demonstrated that neuronal expression of *Jafrac 1*, a *Drosophila* homologue of human *Prx II*(*hPrxII*), is regulated by JNK/FOXO signaling, promotes resistance to oxidative stress, and extends lifespan [27]. In the present work, we studied whether JNK/FOXO signaling can control the expression of *dmsrA* in the presence or absence of exogenous stress. We also investigated the role of *dmsrA* in oxidative stress resistance and lifespan extension.

## 2. Materials and methods

### 2.1. Drosophila culture and stocks

*Drosophila melanogaster* were kept at 25 °C and cultured using standard methods. Wild-type *Oregon-R*, *Arm-Gal4* (ubiquitous), *Elav-GS-Gal4* (pan-neuron Gene Switch), *GMR-Gal4* (eye), *UAS-DJNK-DN*, and *UAS-dFOXO* flies were obtained from the Bloomington Stock Center (Bloomington, IN). *Dcg-Gal4* was a gift from J. Suh. $dFOXO^{21}$/*TM6B* and $dFOXO^{25}$/*TM6B* lines [28] were provided by E. Hafen. *UAS-dmsrA Ri* was obtained from the Vienna Drosophila RNAi Center (VDRC, Vienna, Austria). *UAS-dmsrA* transgenic flies were generated by P-element mediated germline transformation with the *dmsrA* coding sequences.

### 2.2. Paraquat treatment

To test the resistance of *Drosophila* to oxidative stress, adult flies (5 days old) were exposed in 20 mM paraquat. The flies were starved for 6 h in vials containing 1 ml of 1% agar and transferred to vials containing a 22 mm filter paper disks soaked with 20 mM paraquat (methyl viologen, Sigma Chemical Co, St Louis, MO) in a 5% sucrose solution. The number of dead flies was scored after 24 h.

### 2.3. Semi-quantitative reverse transcription-PCR analysis

First strand cDNA was generated using 2 μg of total RNA, the oligo(dT) primer and SuperScript™ III reverse transcriptase (Invitrogen, Carlsbad, CA). 1 μl of the cDNA was used for each PCR reaction (Fig. 3B). PCR conditions were as follows: 94 °C for 5 min, 26 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min using a Thermal Cycler (Applied Bioscience). PCR products were resolved in 1.2% agarose gels and visualized by ethidium bromide staining. The *rp49* gene was used as the control.

### 2.4. Quantitative RT-PCR analysis

Twenty flies were collected for RNA preparation. Total RNA was extracted using the easy-BLUE (TM) reagent (iNtRON biotechnology,

Seoungnam, Korea). All RNA samples were treated with RNase-free DNase (Promega, Madison, WI). cDNA was synthesized using a SuperScript™ III First-Strand Synthesis System (Invitrogen). For quantitative RT-PCR analysis, ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster, CA) and SyberGreen PCR Core reagents (Applied Biosystems) were used. mRNA levels were expressed as the relative fold change against the normalized *rp49* mRNA. The comparative cycle threshold (Ct) method (User Bulletin 2, Applied Biosystems) was used to analyze the data.

*2.5. Western blot analysis*

Antibodies against JNK (Santa Cruz Biotechnology, Santa Cruz, CA) and phospho-JNK (Cell Signaling Technology, Beverly, MA) were used to detect JNK activation. A β-actin antibody (Abcam, Cambridge, USA) was used as the internal control. Total protein from 30 fly heads was prepared by lysing in RIPA buffer (50 mM Tris–Cl pH 7.5, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% DOC) and precipitating with acetone. Western blot analyses were performed as described previously [29].

*2.6. Immunostaining*

An antibody against *Drosophila* FOXO (dFOXO) (a gift from O. Puig) was used to detect dFOXO translocation to the nucleus. For immunostaining, larval fat bodies from third instar larvae were dissected and fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. Samples were incubated with dFOXO antibody (1:500) overnight at 4 °C and with Alexa Fluor 594-conjugated anti-rabbit IgG (1:200, Molecular Probes) for 2 h at room temperature. The tissues were mounted in Vectasheild mounting medium with DAPI (Vector Laboratories, Burlingame, CA) to stain DNA. Fluorescence images were acquired using an Axiovert 200 M microscope (Carl Zeiss, Oberkochen, Germany).

*2.7. Lifespan assay*

For longevity experiments, 1–2 day old adult male or female flies were collected and placed in vials (10 flies per vial). The flies were moved to every 3–4 days to fresh medium containing 200 μM RU486 (Sigma Chemical Co, St Louis, MO) and deaths were recorded. The starting population for each genotype was 50 flies. Three replicates were tested at 22 °C.

*2.8. Scanning Electron Microscopy (SEM)*

Flies were anaesthetized, mounted on the stage, and observed using the Leo 1455VP Environmental Scanning Electron Microscope (KBSI, Korea) in the low vacuum mode.

## 3. Results

*3.1. Overexpression of dmsrA suppresses oxidative stress-induced lethality and extends lifespan*

We measured changes in *dmsrA* mRNA levels in response to treatment with paraquat, an oxidative stress-inducing compound. Levels of *dmsrA* mRNA increased after exposure to 20 mM paraquat for 24 h in the real-time RT-PCR analysis (Fig. 1A). To test the effect of *dmsrA* on oxidative stress, we generated transgenic flies carrying an inducible *dmsrA* gene. When *dmsrA* was expressed ubiquitously, using *Arm-Gal4*, oxidative stress-induced lethality declined (Fig. 1B). Increased expression of the *msrA* mRNA in *Arm-Gal4/UAS-dmsrA*, as compared to the control, was verified by RT-PCR (Supplementary Fig. 1A). Next, we determined the ability of *dmsrA* to increase viability of *Drosophila* in the presence of paraquat-induced oxidative stress. Median survival rate, following exposure to paraquat, was higher for flies overexpressing *dmsrA* (*Arm-Gal4/UAS-dmsrA*) than for the *Arm-Gal4* or *Arm-Gal4/+;UAS-dmsrA Ri/+* flies following exposure to paraquat (Fig. 1C). When neuronal dmsrA was overexpressed (*ElavGS-Gal4/+;UAS-dmsrA/+* or *ElavGS-Gal4/Y;UAS-dmsrA/+*) by adding RU486 to activate the Elav gene switch (ElavGS), lifespan (females, Fig. 2A and male, Fig. 2B, respectively) was extended compared with control. Increased expression levels of the dmsrA mRNA were observed in *ElavGS-Gal4/+;UAS-dmsrA/+* flies treated with RU486 compared to the untreated control flies (Supplementary Fig. 1B). We showed that the *Drosophila* homolog of human *msrA* (*dmsrA*) confers resistance to oxidative stress and that antioxidant function of *dmsrA* may be linked to the increased survival. Moreover, expression of dmsrA plays a role in determining *Drosophila* lifespan.

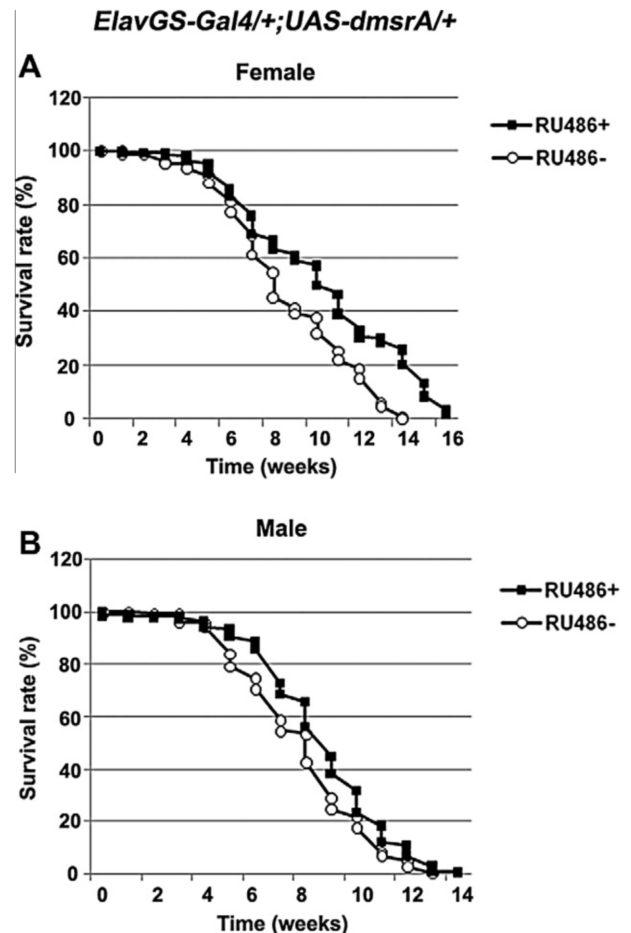
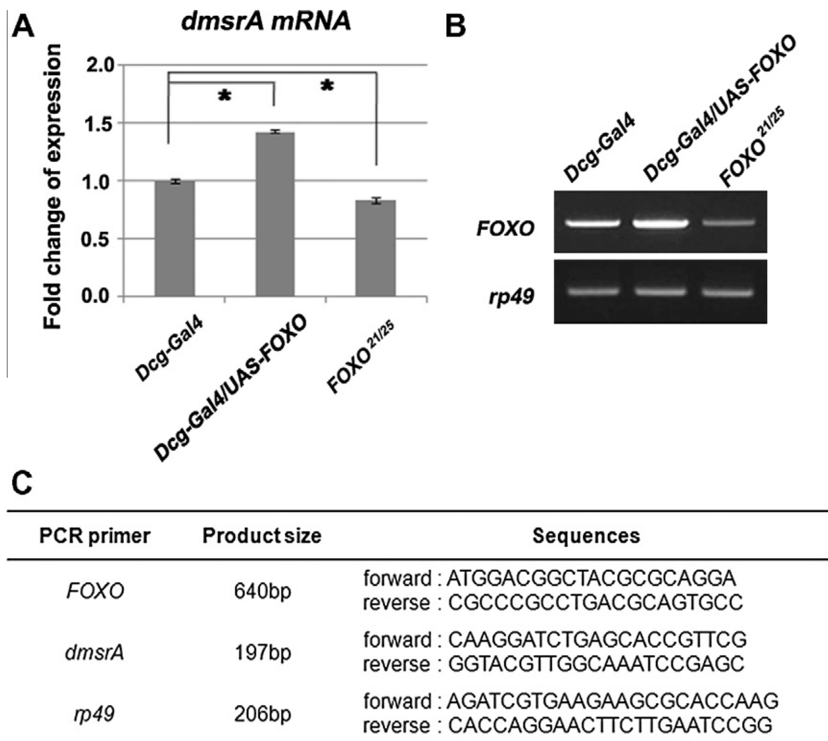



**Fig. 2.** Overexpression of *dmsrA* in neurons extends lifespan in *Drosophila*. Flies carrying *ElavGS-Gal4/+;UAS-dmsrA/+* or *ElavGS-Gal4/Y;UAS-dmsrA/+* were treated RU486 to activate the Elav gene switch (ElavGS) and express *dmsrA*. Flies expressing *dmsrA* had extended lifespan in females (A) and males (B) compared to controls without RU486, in which *dmsrA* was not overexpressed (Log rank test, *ElavGS-gal4* versus *ElavGS- dmsrA/+*, *Gal4/+;UAS-dmsrA/+*, $\chi^2$ = 27.2515, $P$ < 0.0001; *ElavGS-Gal4/Y* versus *ElavGS-Gal4/Y;UAS-* $\chi^2$ = 9.2798, $P$ < 0.0023).

*3.2. FOXO signaling regulates dmsrA expression*

We previously showed that oxidative stress activated JNK and FOXO in *Drosophila* neurons [26]. In that study, we demonstrated that *Jafrac 1*, a *Drosophila* homologue of human *Prx II*(*hPrxII*), functions as a downstream effector of JNK/FOXO signaling in neurons, increases stress resistance, and extends lifespan. Recently, *msrA-1* was found to be regulated by DAF-16/FOXO3a in C. *elegans*




| PCR primer | Product size | Sequences |
|---|---|---|
| *FOXO* | 640bp | forward : ATGGACGGCTACGCGCAGGA<br>reverse : CGCCCGCCTGACGCAGTGCC |
| *dmsrA* | 197bp | forward : CAAGGATCTGAGCACCGTTCG<br>reverse : GGTACGTTGGCAAATCCGAGC |
| *rp49* | 206bp | forward : AGATCGTGAAGAAGCGCACCAAG<br>reverse : CACCAGGAACTTCTTGAATCCGG |

**Fig. 3.** *dmsrA* is a target gene of FOXO in fat body cells. (A) In the real-time RT-PCR analysis, overexpression of *FOXO* in fat body cells (*Dcg-Gal4/UAS-foxo*) increased the expression level of *dmsrA* compared to the *Dcg-Gal4* control while the expression level of *dmsrA* was reduced in the fat body cells of $FOXO^{21/25}$ mutant animals. Data is represented as mean ± S.E. from three independent experiments (*$P < 0.05$, Student's *t*-test). (B) Expression of *FOXO* was regulated by the *FOXO* overexpression and $FOXO^{21/25}$ mutants. (C) Sequences of primers used for PCR are listed.

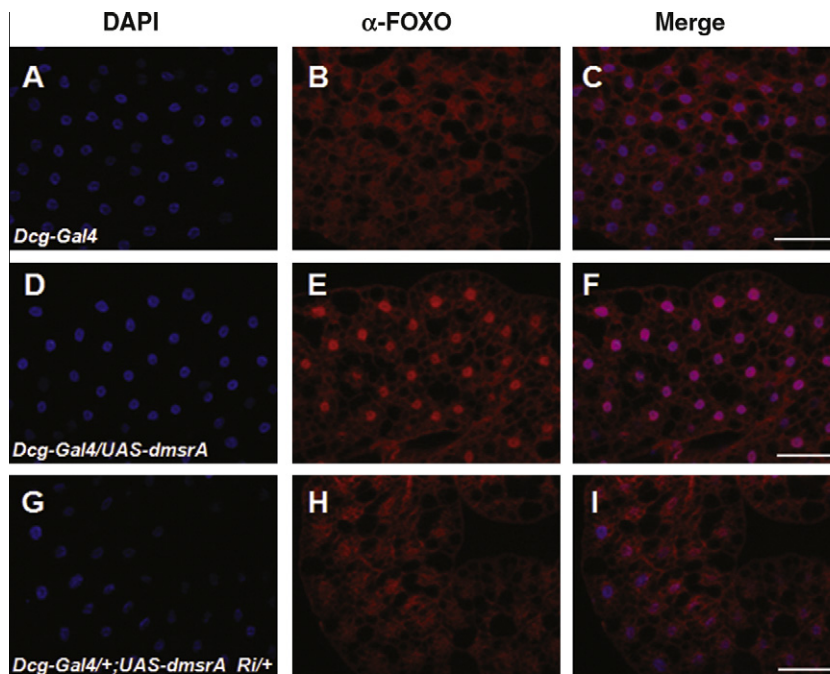



**Fig. 4.** Subcellular localization of FOXO in fat body cells of *dmsrA* overexpression and inhibition. When *dmsrA* was overexpressed in the fat body cells (*Dcg-Gal4/UAS-dmsrA*), FOXO was primarily localized in the nucleus (D–F). In control cells (*Dcg-Gal4*) FOXO was found equally in the nucleus and cytoplasm (A–C). In contrast, when *dmsrA* was inhibited in fat body cells (*Dcg-Gal4/+;UAS-dmsrA Ri/+*) FOXO did not relocalize to the nucleus (G–I), but instead resembled the *Dcg-Gal4* control (A–C) (scale bar, 100 μm).

[19]. To investigate whether FOXO signaling regulates *dmsrA* expression in *Drosophila*, we overexpressed *dFOXO* in fat body cells using *Dcg-Gal4*. Overexpression of *FOXO* in fat body cells (*Dcg-Gal4/UAS-FOXO*) increased the expression level of *dmsrA* compared to the *Dcg-Gal4* control while the expression level of *dmsrA* was reduced in the fat body cells of $FOXO^{21/25}$ mutant flies (Fig. 3A). Expression of *FOXO* was verified to be regulated by the *FOXO* overexpression and $FOXO^{21/25}$ mutants (Fig. 3B). These results indicate that expression of *dmsrA* is regulated by dFOXO in *Drosophila*.

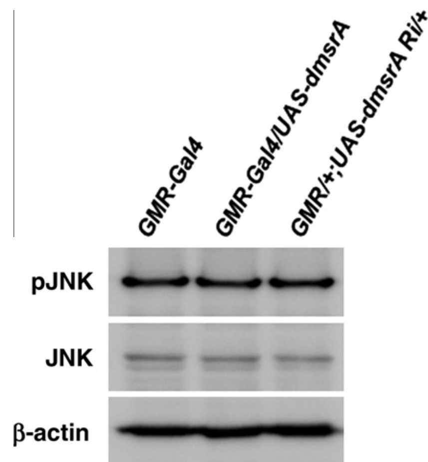



**Fig. 5.** *dmsrA* does not activate JNK in *Drosophila* eyes. Neither overexpression nor inhibition of *dmsrA* in eyes (*GMR-Gal4/UAS-dmsrA* and *GMR/+;UAS-dmsrA Ri/+*) activated JNK in the Western blot analysis.

### 3.3. dmsrA increases nuclear translocation of dFOXO

In our previous study, overexpression of *Jafrac1* in neurons suppressed JNK activation in the presence of oxidative stress [27]. However, unlike *Jafrac1*, *dmsrA* overexpression did not suppress JNK phosphorylation in response to oxidative stress (data not shown). We speculated that, unlike *Jafrac1*, *dmsrA* not only functions as a effector protein, but also has other regulatory functions. We found that dFOXO expression was equal in the cytoplasm and the nucleus of wild type larval fat body cells. However, overexpression of *dmsrA* in fat body cells (*Dcg-Gal4/UAS-dmsrA*) increased nuclear localization of dFOXO. Inhibition of *dmsrA* (*Dcg-Gal4/+;UAS-dmsrA Ri/+*) prevented nuclear localization of dFOXO (Fig. 4). We observed increased expression levels of the *dmsrA* mRNA *in Dcg-Gal4/UAS-dmsrA* as compared to control flies (Supplementary Fig. 1C). This result suggests a positive feedback loop between antioxidant enzyme dmsrA and transcriptional factor dFOXO may amplify resistance to oxidative stress and extend lifespan in flies.

### 3.4. Manipulation of dmsrA levels did not activate JNK in Drosophila eyes

Overexpression or inhibition of *dmsrA* in fly eyes (*GMR-Gal4/UAS-dmsrA* or *GMR-Gal4/+;UAS-dmsrA Ri/+*) did not change the level of phopho-JNK or JNK (Fig. 5). Overexpression of *dmsrA* in *Drosophila* eye, using a GMR driver (*GMR-Gal4/UAS-dmsrA*), resulted in increased irregularity and a rough eye phenotype. As shown in Supplementary Fig. 2B, eye size and cell number was increased in eyes overexpressing *dmsrA*. Flies with *JNK* reduction and overexpression of *dmsrA* (*GMR-Gal4/UAS-dmsrA;UAS-JNK-DN/+*) (Supplementary Fig. 2C) had a phenotype similar to that caused by *dmsrA* overexpression (*GMR-Gal4/UAS-dmsrA*) (Supplementary Fig. 2B). These results indicate that JNK signaling may not regulate *dmsrA* expression in *Drosophila*.

## 4. Discussion

In this study, we demonstrated that dFOXO is required for the expression of *dmsrA* in flies. We also showed that overexpression of *dmsrA* reduces oxidative stress-induced lethality. Furthermore, ubiquitous overexpression of *dmsrA* extended the lifespan. These results support the hypothesis that the FOXO pathway protects *Drosophila* from oxidative stress and extends lifespan by induction of antioxidant genes, including *dmsrA*. We did not find the decreased resistance to oxidative stress in flies with UAS-dmsrA Ri. We speculate that other backup and redundant antioxidant systems function to compensate the decreased level of dmsrA. The lack of difference in the sensitivity to oxidative stress between control and flies with UAS-dmsrA Ri is not because dmsrA has not been reduced (Supplementary Fig. 1D).

MsrA is an important antioxidant enzyme that has a role in the maintenance of protein structure and function by repairing oxidized methionine residues [5,6,10]. Although *msrA-1* expression was known to be regulated by the DAF-16/FOXO3a in C. *elegans* [19], we showed that d*msrA* may also be modulated by dFOXO in *Drosophila*.

JNK is known to activate FOXO in mammalian cells and flies [21,24,30]. JNK also plays an important role in cytoprotection and adaptive response to stress [31]. However, in this study, we found that JNK did not directly regulate the expression of *dmsrA*, although it may be the upstream regulator of dFOXO in cellular response to oxidative stress in *Drosophila* [20,21,26,28,32].

We demonstrated for the first time that *dmsrA* increased nuclear translocation of dFOXO. Few reports have shown that the overexpression of antioxidant enzymes might influence the level of transcription of other proteins or enzymes. This finding suggests that the resistance to the oxidative stress and prolonged lifespan caused by *dmsrA* may be due to both its antioxidant activity and of its regulation of dFOXO activity [21,28,33]. Several negative feedback regulations of dFOXO, including the insulin-PI3K-PKB signaling cascade, have been reported [28,33,34]. However, a possible positive feedback loop between dmsrA and dFOXO, which could potentiate the antioxidant activity of dmsrA, has not been studied previously. FOXO provides tolerance against oxidative insults [28]. Under stressful condition such as oxidative stress or starvation, FOXO translocates to the nucleus and activates target genes required for stress protection, damage repair, decreased cellular metabolism and growth arrest [21,28,33].

In conclusion, our findings indicate that *dmsrA* may extend lifespan by acting an antioxidant enzyme and by regulating dFOXO under normal and oxidative stress conditions.

## Acknowledgements

This work was supported by the Grants from the National Research Foundation of Korea (2008-0061669 and 2010-0000846), the KRIBB Research Initiative Program, and the Pioneer Research Program for Converging Technology of the Ministry of Education, Science and Technology, Republic of Korea (Grant No. M107 1118001-08M1118-00110, 2008).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.07.033.

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