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Methionine sulfoxide reductase B in the endoplasmic reticulum is critical for stress resistance and aging in *Drosophila*

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ABSTRACT

Methionine sulfoxide reductase B (MsrB) is an enzyme that repairs oxidatively damaged proteins by specifically reducing methionine-*R*-sulfoxide back to methionine. Three MsrBs, localized in different cellular compartments, are expressed in mammals. However, the physiological roles of each MsrB with regard to its location remain poorly understood. Here, we expressed endoplasmic reticulum (ER)-targeted human MsrB3A (hMsrB3A) in *Drosophila* and examined its effects on various phenotypes. In two independent transgenic lines, both ubiquitous and neuronal expression of hMsrB3A rendered flies resistant to oxidative stress. Interestingly, these flies also showed significantly enhanced cold and heat tolerance. More strikingly, expression of hMsrB3A in the whole body and nervous system extended the lifespan of fruit flies at 29 °C by 43–50% and 12–37%, respectively, suggesting that the targeted expression of MsrB in the ER regulates *Drosophila* lifespan. A significant increase in lifespan was also observed at 25 °C only when hMsrB3A was expressed in neurons. Additionally, hMsrB3A overexpression significantly delayed the age-related decline in locomotor activity and fecundity. Taken together, our data provide evidence that the ER type of MsrB, MsrB3A, plays an important role in protection mechanisms against oxidative, cold and heat stresses and, moreover, in the regulation of fruit fly aging.

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1. Introduction

Oxidation of proteins is associated with aging and a variety of diseases. The sulfur-containing amino acid methionine is readily oxidized to methionine sulfoxide by reactive oxygen species (ROS), but this oxidation can be reversed by an enzymatic reduction system. Methionine sulfoxide reductases (Msrs) are the enzymes responsible for the reduction of methionine sulfoxide to methionine [1–3]. Two distinct Msr families, MsrA and MsrB, with different substrate stereospecificities have evolved for the methionine sulfoxide reduction in proteins. MsrA is specific for the *S*-form of methionine sulfoxide, whereas MsrB only acts on the *R*-form. Msrs are implicated in the regulation of the aging process of organisms due to their functions as protein repair enzymes and antioxidants.

In contrast to a single MsrA gene, there are three MsrB genes in mammals coding for proteins targeted to different cellular compartments [4]. MsrB1 is a selenoenzyme that is present in cytosol and nucleus. MsrB2 is localized to mitochondria. In humans, MsrB3 gives rise to two alternatively spliced forms, MsrB3A and MsrB3B, with only different N-terminal signal peptides. MsrB3A is targeted to the ER, whereas the other form, MsrB3B, is targeted to mitochondria. However, no evidence for alternative splicing of MsrB3 was found in mouse [5]. Instead, mouse MsrB3 contains consecutive N-terminal ER and mitochondrial targeting signals and is targeted only to ER for unknown reasons [5]. Studies are needed to address the physiological roles of each MsrB with regard to its location.

The *Drosophila* genome does not encode a clear ortholog of human MsrB3A (hMsrB3A) [6]. The fruit fly *Drosophila* is an excellent model organism to study the effects of a particular gene on stress response and lifespan. In this work, we report the effects of overexpression of ER-type MsrB, hMsrB3A, on lifespan, stress resistance, locomotor activity, and fecundity in *Drosophila*.

2. Materials and methods

2.1. Drosophila strains

All experimental crosses were performed at 25 °C on standard cornmeal/agar media (6.3% cornmeal, 2.6% yeast, 6.3% molasses, 0.9% agar, 1.4% tegosept and 0.5% propionic acid) under non-crowded conditions. The expression of hMsrB3A in *Drosophila* was achieved using the *GAL4/UAS* transactivation system [7]. To generate *UAS-hMsrB3a* transgenic lines, the coding region of hMsrB3A from a pET28a derivative harboring hMsrB3A [4] was inserted into *EcoRI/NotI* sites of pUAST [7]. The nucleotide sequence

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of the resulting construct was confirmed by DNA sequencing. The construct was co-microinjected into w^{1118} embryos with *P*{Δ2–3} plasmid carrying a transposase-coding gene by a standard procedure, and multiple independent transformants carrying *UAS-hMsrB3a* with no discernable defects in viability and fertility were obtained. Two independent homozygous lines for hMsrB3A with insertions on the second (*UAS-hMsrB3a[6A]*) and third chromosome (*UAS-hMsrB3a[17C]*) were used in this study. *Daughterless* (*da*)-*GAL4* [*w**; *P*{$w^{+mW.hs}$ = *GAL4-da.G32}UH1*] and *elav-GAL4* [*w*, *P*{$w^{+mW.hs}$ = *GawB}elav*^{C155}] driver lines were used to induce expression of the *UAS-hMsrB3a* in the whole body and nervous system, respectively. To minimize effects of genetic background variations, *UAS-hMsrB3a[6A]*, *UAS-hMsrB3a[17C]*, and the two *GAL4* driver lines were outcrossed six times to a w^{1118} strain (stock number 5905) obtained from the Bloomington Stock Center. All described assays were performed with progeny generated from crosses of the specified *GAL4* females with *UAS-hMsrB3a* or w^{1118} males (for the driver control).

2.2. MsrB enzyme assay

Thirty flies were homogenized in 100 μl PBS containing complete protease inhibitors (Roche). The homogenates were centrifuged at 12,000*g* for 10 min at 4 °C, and the supernatant was subjected to MsrB enzyme assay as described previously [8]. Briefly, the reaction mixture (100 μl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 20 mM dithiothreitol, 200 μM dabsylated methionine-*R*-sulfoxide, and 200 μg crude protein. The reaction was carried out at 37 °C for 30 min and the reaction product, dabsyl-Met, was analyzed by an HPLC procedure [8].

2.3. Construct for subcellular localization and confocal fluorescence microscopy

A DNA fragment encoding GFP-fused hMsrB3A from pS3A-GFP-MsrB3 [4] was inserted into *XhoI*/*NotI* sites of pMK33. The resulting construct, where GFP is inserted in between the N-terminal 31 amino acids and the rest of hMsrB3A, was named pMK-GFP-hMsrB3A. Culture and transfection of *Drosophila* Schneider 2 (S2) cells were carried out as previously described [9]. S2 cells transiently transfected with pMK-GFP-hMsrB3A were induced to express GFP-hMsrB3A by the addition of $CuSO_4$ to a final concentration of 0.7 mM. Two days after induction, cells were incubated with 1 μM ER-Tracker (Molecular Probes) for 30 min at 25 °C, and mounted in Vectashield medium (Vector Laboratories). The cells were imaged using a Zeiss LSM5 EXCITER confocal laser-scanning microscope.

2.4. Stress resistance tests

UAS-hMsrB3a[6A] and *UAS-hMsrB3a[17C]* lines were mated to isogenic *GAL4* drivers, and progenies of the cross were raised to adulthood at 25 °C. As a control, crosses were also made between isogenic *GAL4* drivers and w^{1118}. Flies of each genotype were collected in mixed-sex groups within 24 h of eclosion and aged at 29 °C until testing. Cohorts of 10-day-old male or female flies (20 per vial) were used for various stress tests, each of which was repeated at least five times. Resistance to oxidative stress was assayed as described previously [10] with modifications. To avoid the variation arising from differential ingestion rates of paraquat, flies of each genotype were first starved for 4 h in empty vials, and then transferred to a solid medium containing 1% agarose, 2% sucrose, and 10 mM paraquat (Sigma–Aldrich). Survivors were counted periodically at 29 °C. Cold stress test was performed as described previously [11] with some modifications. Cohorts of 20 flies of each genotype were placed into food vials containing the standard cornmeal/agar medium and kept for 8 h at 4 °C, except for a 16 h recovery period at 29 °C daily. Animals were scored for survival every day. Thermal stress test was performed as described previously [12]. Cohorts of 20 flies of the indicated genotypes were transferred to vials with solid medium containing 1% agarose and 2% sucrose, and scored for survivors at 37 °C.

2.5. Lifespan assays

Transgenic *da-GAL4/UAS-hMsrB3a* and *elav-GAL4/UAS-hMsrB3a* flies, and control *da-GAL4/+* and *elav-GAL4/+* flies were reared to adulthood at 25 °C. Newly eclosed flies of each genotype were collected within 24 h, and cohorts of mated male or female flies (20 per vial) were maintained at 25 °C or 29 °C, and transferred to the fresh cornmeal/agar medium every 3 days. Survivors were counted daily until all the flies had died. A total of 100 flies for each genotype were tested for lifespan at 29 °C. A total of 140 and 440 flies driven by *elav-GAL4* and *da-GAL4*, respectively, for each genotype were tested for lifespan at 25 °C.

2.6. Food intake assay

Twenty-five male or female flies (10-day-old) of each genotype were placed into vials with solid medium containing 1 mg/ml Brilliant Blue dye, 1% agarose, and 2% sucrose at 29 °C for 20 h. Groups of five flies were homogenized in distilled water, and the resulting extracts were used to determine the dye concentration by measuring the absorbance at 630 nm.

2.7. Climbing assay

The climbing assay was performed as described previously [13,14] with some modifications. Ten flies of each genotype aged for 3, 10, and 20 days at 29 °C were placed into an empty vial with a line drawn 2 cm from the bottom of the vial. Flies were recovered from anesthesia for 30 min and gently tapped to the bottom of the vial. The number of flies above the 2-cm mark was counted after 20 s climbing. Ten trials were performed for each time point. The experiment was performed at room temperature under red light at dark room, and repeated five times for each genotype.

2.8. Fecundity assay

Four virgin female *da-GAL4/+*, *da-GAL4/UAS-hMsrB3a[6A or 17C]*, *elav-GAL4/+*, or *elav-GAL4/UAS-hMsrB3a[6A* or *17C]* flies were placed together with the same number of 1-day-old w^{1118} males in a cage with egg laying medium at 25 °C. The egg-laying medium was replaced with fresh medium every day and the number of eggs laid in a 24-h period was counted for every 5 day in five independent experiments of each genotype.

2.9. Statistical analyses

Survival curves of stress tests and lifespan assays were analyzed with log-rank test. The significance of food intake, climbing activity and fecundity was analyzed by ANOVA with supplementary Dunnett's test. Prism 5 software (GraphPad) was used for statistical analysis of data. A *P* value of <0.05 was considered significant.

3. Results

3.1. ER localization of human MsrB3A in Drosophila

In human cells, hMsrB3A is localized to the ER [4]. Thus, we first tested whether hMsrB3A can localize in the ER of *Drosophila* cells.

The GFP-hMsrB3A fusion protein colocalized with ER-Tracker (Supplementary Fig. S1), demonstrating that hMsrB3A resides in the ER in *Drosophila* cells as does in human cells.

3.2. Overexpression of hMsrB3A in Drosophila

We generated *UAS-hMsrB3a* transgenic flies to overexpress hMsrB3A using the *UAS/GAL4* system [7]. Two independent lines, *UAS-hMsrB3a[6A]* and *UAS-hMsrB3a[17C]*, were used for further study. Due to the neuron-specific expression, lower levels of hMsrB3A were observed in *elav-GAL4/UAS-hMsrB3a* flies, compared to those in *da-GAL4/UAS-hMsrB3a* flies (Supplementary Fig. S2A). No expression of hMsrB3A was detected in the driver controls (*da-GAL4/+* and *elav-GAL4/+*). Consistent with the Western blot data, a sixfold increase in MsrB activity was observed in extracts from *da-GAL4/UAS-hMsrB3a* flies, while a twofold increased activity was detected in extracts from *elav-GAL4/UAS-hMsrB3a* flies (Supplementary Fig. S2B).

3.3. Overexpression of hMsrB3A increases oxidative stress resistance in Drosophila

We examined the resistance of hMsrB3A-overexpressing flies to paraquat-induced oxidative stress at 29 °C, a temperature at which the activity of the *GAL4/UAS* system is enhanced [15]. Prior to this experiment, we assayed food intake of hMsrB3A-overexpressing flies and compared intake with that of the driver controls (Supplementary Fig. S3). Increased food intake was observed in *da-GAL4*-driven hMsrB3A-overexpressing flies but not statistically significant. No different food intake was also observed between *elav-GAL4*-driven hMsrB3A-overexpressing and control flies.

When treated with paraquat, flies that ubiquitously overexpressed hMsrB3A by the *da-GAL4* driver showed increased survival in both sexes, compared to the driver controls (Fig. 1A and B, and Table 1). Their mean survival was increased by 25–63% in males and by 33% in females, relative to the controls. Increased resistance to oxidative stress was also observed when hMsrB3A was expressed in neurons using the *elav-GAL4* driver. (Fig. 1C and D, and Table 1), confirming the specificity of hMsrB3A-induced resistance to oxidative stress. The neuronal expression of hMsrB3A led to an average increase in mean survival by 21% in males and by 67% in females. Together, these results support a protective role for hMsrB3A as an antioxidant.

3.4. Overexpression of hMsrB3A increases cold and heat tolerance in Drosophila

In *Arabidopsis*, it was previously found that plant ER-targeted MsrB3 is expressed in a cold-responsive manner, and confers tolerance to plants in response to cold stress [16,17]. Thus, it was of interest to determine whether the ER-type hMsrB3A may have a potential function in response to cold in *Drosophila*. We tested the sensitivity of hMsrB3A transgenic flies to cold stress at 4 °C for 8 h daily after 16-h recovery at 29 °C. Interestingly, in response to cold stress, flies overexpressing hMsrB3A in the whole body showed a twofold increase in mean survival in both sexes relative to the driver controls (Fig. 2A and B, and Table 1). Similar effects of hMsrB3A on cold stress resistance were observed when overexpressed in the nervous system; a twofold increase in mean survival of hMsrB3A-expressing flies was observed in both sexes, compared to the driver controls (Fig. 2C and D, and Table 1). Together, the data demonstrated that the ER-targeted expression of MsrB3 increases tolerance to cold stress in *Drosophila*.

Next, we examined the tolerance of hMsrB3A transgenic flies to heat stress by incubating them at 37 °C. Similar to the protective effect of hMsrB3A against cold stress, the hMsrB3A-expressing flies were also resistant to heat stress. Both males and females of *da-GAL4/UAS-hMsrB3a[6A]* and *da-GAL4/UAS-hMsrB3a[17C]* showed an increase of 38% in mean survival at 37 °C (Supplementary Fig. S4 and Table 1). Also, *elav-GAL4/UAS-hMsrB3a[6A]* and *elav-GAL4/UAS-hMsrB3a[17C]* exhibited an increase of 33–46% in mean survival in males and 30–35% in females (Supplementary Fig. S4 and Table 1). Together, these results demonstrated that hMsrB3A

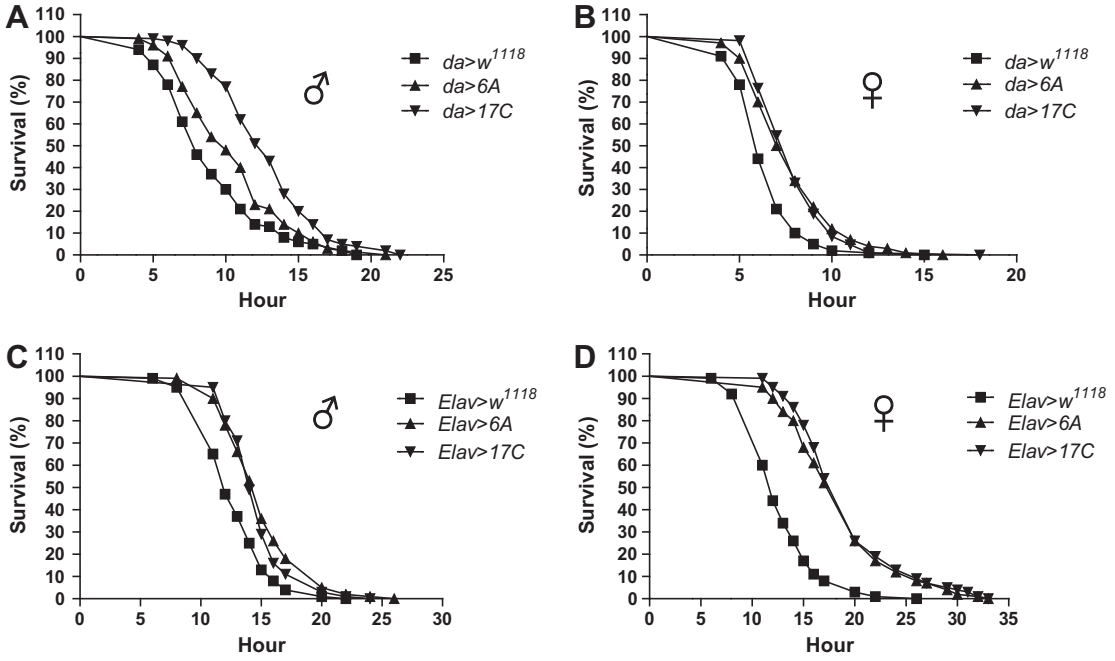

Fig. 1. Increased resistance to oxidative stress by hMsrB3A expression. Ten-day-old male and female flies overexpressing hMsrB3A driven by *da-GAL4* (A and B) and *elav-GAL4* (C and D), together with age-matched driver control flies, were exposed to 10 mM paraquat and scored for survivorship (n = 100/sex/genotype). Percent survival at different times is shown. Median survival for flies of each indicated genotype and statistical significance are indicated in Table 1.

Table 1
Statistical analysis of survival curves.

Test	Line		Median survival			Statistical analysis (p-value)[a]	
			w^{1118}	[6A]	[17C]	[6A]	[17C]
Oxidative stress	*da-Gal4*	Male	8 h	10 h	13 h	0.01111	<0.0001
		Female	6 h	7.5 h	8 h	0.01111	<0.0001
	elav-Gal4	Male	12 h	15 h	14 h	<0.0001	<0.0001
		Female	12 h	20 h	20 h	<0.0001	<0.0001
Cold stress	*da-Gal4*	Male	11.5 d	23 d	23 d	<0.0001	<0.0001
		Female	7 d	14 d	15 d	<0.0001	<0.0001
	elav-Gal4	Male	7 d	13 d	13 d	<0.0001	<0.0001
		Female	10 d	18 d	15.5 d	<0.0001	<0.0001
Heat stress	*da-Gal4*	Male	8 h	11 h	11 h	<0.0001	<0.0001
		Female	8 h	11 h	11 h	<0.0001	<0.0001
	elav-Gal4	Male	24 h	32 h	35 h	<0.0001	<0.0001
		Female	23 h	29.5 h	31 h	<0.0001	<0.0001
Lifespan (at 29 °C)	*da-Gal4*	Male	22 d	27 d	33 d	<0.0001	<0.0001
		Female	21 d	30 d	30 d	<0.0001	<0.0001
	elav-Gal4	Male	26 d	29 d	28 d	<0.0001	0.0232
		Female	25.5 d	35 d	35 d	<0.0001	<0.0001
Lifespan (at 25 °C)	*da-GAL4*	Male	47.5 d	45 d	53 d	0.0985	<0.0001
		Female	47.5 d	46.5 d	55 d	0.0873	<0.0001
	elav-Gal4	Male	40 d	51 d	50 d	<0.0001	<0.0001
		Female	51 d	70 d	70.5 d	<0.0001	<0.0001

[a] Statistical analysis using log-rank test.

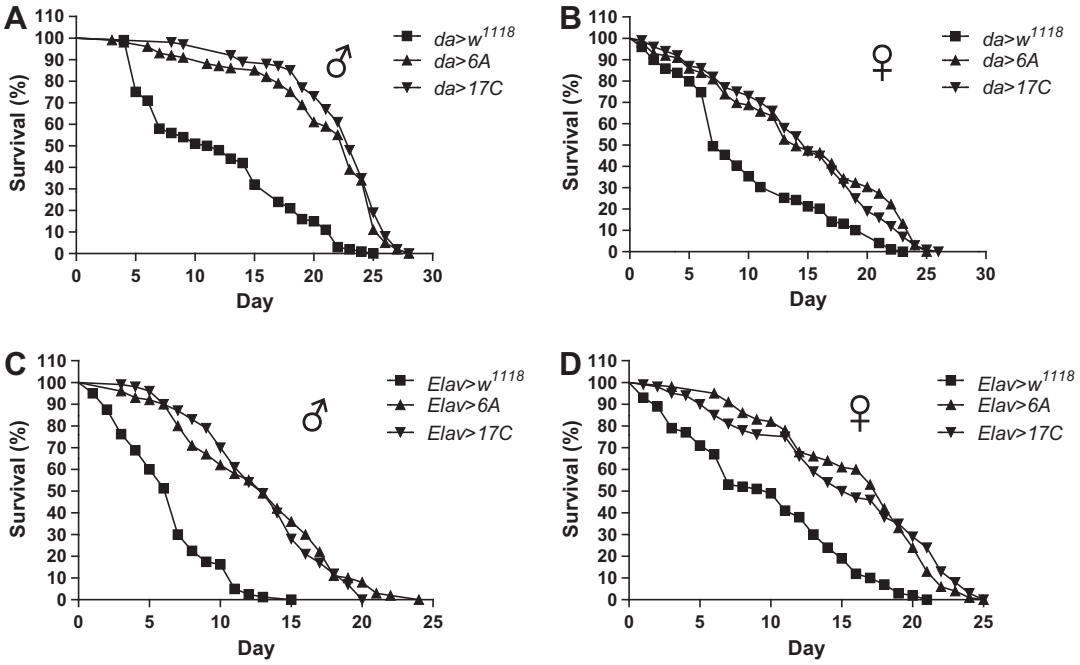

Fig. 2. Increased resistance to cold stress by hMsrB3A expression. Ten-day-old control and hMsrB3A-overexpressing flies of the indicated genotypes were scored for survival in response to cold stress at 4 °C (n = 100/sex/genotype). Median survival for flies of each indicated genotype and statistical significance are indicated in Table 1.

expressed in the ER plays an essential role in protection against heat stress as well as cold stress in *Drosophila*.

3.5. Overexpression of hMsrB3A extends lifespan of Drosophila

Next, we determined whether hMsrB3A expression has any beneficial effects on the lifespan of adult flies. We first performed lifespan assays at 29 °C where enhanced activity of *GAL4/UAS* system is exerted [15]. The ubiquitous expression of hMsrB3A in the whole body with the *da-GAL4* driver extended lifespan by 50% in males and 43% in females, as compared to the driver controls (Fig. 3A and B, and Table 1). Similarly, neuron-specific expression of hMsrB3 using the *elav-GAL4* driver led to a significant increase in lifespan of males and females by 12% and 37%, respectively (Fig. 3C and D, and Table 1). We further assayed the lifespan of hMsrB3A transgenic flies at 25 °C. Overexpression of hMsrB3A in neurons resulted in 27% and 38% increase in the lifespan of males and females, respectively (Supplementary Fig. S5 and Table 1). However, the lifespan of flies was not significantly affected when hMsrB3A was ubiquitously expressed in the whole body (Supplementary Fig. S5 and Table 1); *hMsrB3a[17C]* transgenic flies showed a significant increase in lifespan of males and females, whereas

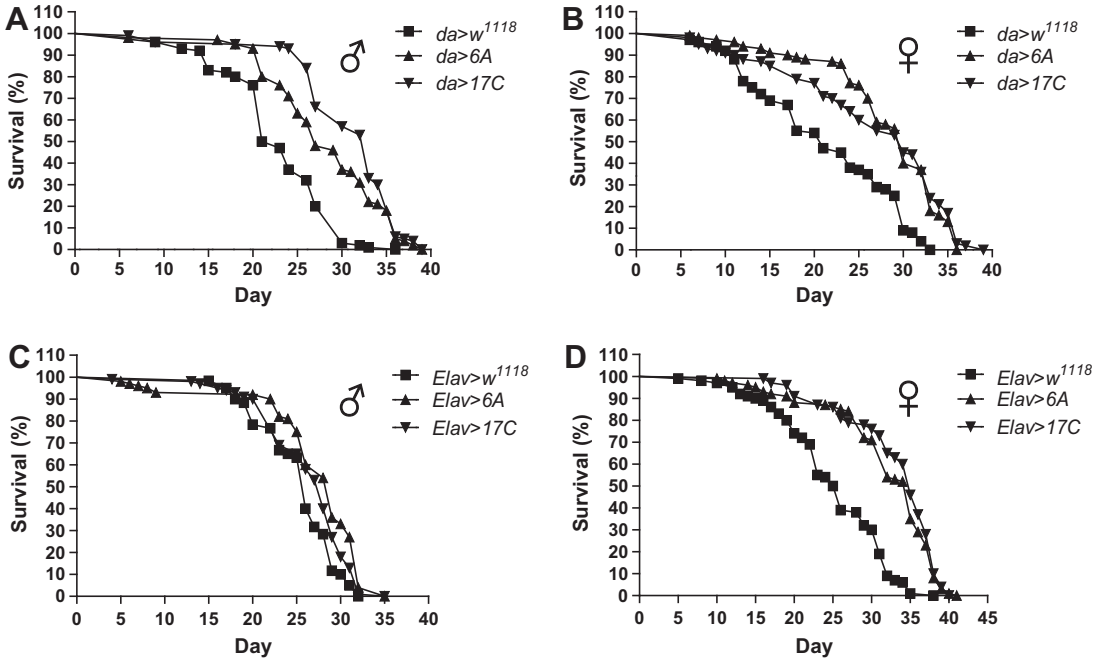

Fig. 3. Lifespan extension by expression of hMsrB3A. Survival of hMsrB3A in *da-GAL4/UAS-hMsrB3a* (A and B), *elav-GAL4/UAS-hMsrB3a* (C and D), and matched driver control flies of both sexes was recorded on standard food at 29 °C (n = 100/sex/genotype). Median survival for flies of each indicated genotype and statistical significance are indicated in Table 1.

hMsrB3a[6A] transgenic flies showed no difference compared with the control flies. Taken together, our data indicate that hMsrB3A can positively regulate the lifespan of *Drosophila* and that its expression in neurons is sufficient to delay aging of flies.

3.6. Overexpression of hMsrB3A protects against age-related locomotor dysfunction

To examine the effect of neuronal expression of hMsrB3A on locomotor behavior of flies, we measured their climbing activity during the aging process (Fig. 4). The climbing assay showed an age-dependent decline in climbing ability. However, 10-day-old flies expressing hMsrB3A in neurons displayed a significant increase in climbing ability by 36–40% and 12–19% in males and females, respectively, compared to the controls. Similarly, this neuron-specific hMsrB3A expression significantly improved the performance of 20-day-old male and female flies by 25–34% and 34–54%, respectively. However, no significant difference in climbing ability was observed at day 3 of adulthood between *elav-GAL4/UAS-hMsrB3a* and *elav-GAL4/+* driver control. These results suggest that ER-targeted expression of hMsrB3A protects against progressive motor deficits during the aging process.

3.7. hMsrB3A-expressing flies display an increased fecundity

Finally, we examined whether the ER-targeted hMsrB3A expression affects fecundity, as defined by the number of eggs laid by females [18]. We counted the number of eggs produced per female of different ages (5-, 10-, 15-, 20-, and 25-day-old animals) in one day (Supplementary Fig. S6). Overall, the fecundity declined with aging. However, hMsrB3A-expressing flies in the whole body in all different age groups showed a significant increase in fecundity, compared to the age-matched control animals. Also, 5- and 10-day-old females expressing hMsrB3A in the nervous system showed an increase in fecundity, compared to the age-matched controls. However, no dramatic changes in fecundity were observed in 15-, 20-, and 25-day-old animals expressing hMsrB3A in the neurons. Collectively, overexpression of hMsrB3A increased the egg production of female flies, suggesting delayed onset of the decline in the overall reproductive vigor.

4. Discussion

Both MsrA and MsrB enzymes have been implicated in resistance to oxidative stress by scavenging ROS via the function of cyclic reduction of oxidized methionine residues. The antioxidant defense role of MsrA has been evidenced in different model organisms [19–21]. In the case of MsrB, its antioxidant function has also been demonstrated at the cellular level in yeast and mammals [22,23]. The effects of cytosolic or mitochondrial MsrB overexpression in *Drosophila* on the protection against oxidative stress have previously been reported [24,25]. The effect of cytosolic form of MsrB seems different according to the source of protein. Neuronal expression of cytosolic *Drosophila* MsrB has no effect on resistance to paraquat-induced oxidative stress [24]. By contrast, the expression of cytosolic mouse MsrB1 in the nervous system shows the positive effect on resistance to paraquat- and H_2O_2-induced oxidative stress [25]. Neuronal expression of the mitochondrial form of MsrB, mouse MsrB2, does not increase resistance to paraquat-induced oxidative stress [24]. Presently, the ER-targeted expression of MsrB in *Drosophila* had a positive effect on resistance to oxidative stress; hMsrB3A expression in the both nervous system and whole body increased resistance to paraquat-induced oxidative stress. The findings suggest that in fruit flies, methionine-*R*-sulfoxide reduction in the ER may be important for protection against oxidative stress.

This study highlights a critical role of ER-localized MsrB in protection against cold and heat stresses in *Drosophila*. The survival of hMsrB3A transgenic flies in response to cold stress was markedly increased (more than twofold), compared to control flies. In addition to cold stress resistance, the ER-targeted expression of hMsrB3A also conferred tolerance to heat stress on flies. The

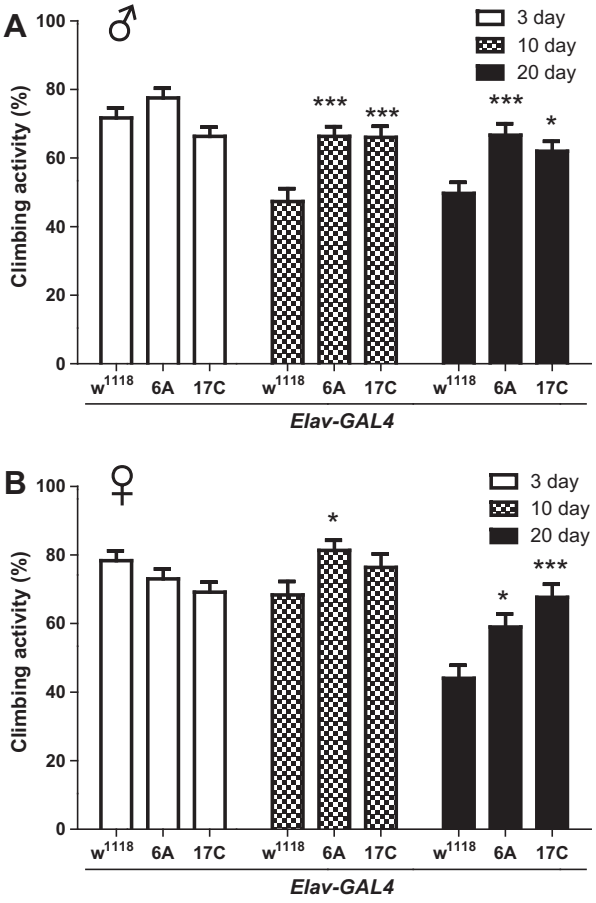

Fig. 4. Expression of hMsrB3A in neurons delays loss of climbing ability with age in flies. Flies with hMsrB3A overexpression driven by pan-neuronal *elav-GAL4* were aged for the indicated day at 29 °C, and analyzed for their climbing activity at room temperature, as compared to age-matched driver control flies. Data are shown as mean ± SEM from five independent experiments with cohorts of 10 flies per genotype per sex (*$P < 0.05$; ***$P < 0.001$, ANOVA with supplementary Dunnett's test).

survival of hMsrB3A-overexpressing flies was significantly increased (more than 30%) under high temperature of 37 °C, compared to the controls. These findings suggest that ER-targeted MsrB3 plays an important role in thermal tolerance and that a methionine-*R*-sulfoxide reduction pathway in the ER can regulate temperature responses in animals. It would be of interest to investigate whether MsrA or other types of MsrB (cytosolic or mitochondrial forms) can play a role in tolerance to thermal stress in animals. Also, studies are needed concerning the regulation of thermal responses mediated by MsrB3, including searching for target proteins of MsrB3 upon cold or heat stresses.

Previous reports have demonstrated a positive role of methionine-*S*-sulfoxide reduction in delaying fruit fly aging [21,26]. In contrast, methionine-*R*-sulfoxide reduction has no function yet in regulation of fruit fly aging. Previously, overexpression of cytosolic *Drosophila* MsrB, mouse selenoenzyme MsrB1, or mitochondrial mouse MsrB2 had no effect on the lifespan of *Drosophila* at all, suggesting that MsrA and MsrB have different effects on aging in fruit flies [24,25]. However, the present study clearly demonstrated that ER-targeted overexpression of MsrB3 extended the *Drosophila* lifespan. The collective data support the view that methionine-*R*-sulfoxide reduction in the ER, but not in cytosol and mitochondria, can be a determinant for regulating the fruit fly lifespan.

The temperature of 29 °C should be a stress condition for survival, while 25 °C is a normal condition. As mentioned earlier, hMsrB3A overexpression led to increased tolerance to heat stress in *Drosophila*. Thus, the extension of the lifespan of flies overexpressing hMsrB3A, particularly at 29 °C, may also be attributed to the increased heat stress tolerance. This could be one possibility to explain the different effects of hMsrB3A overexpression in the whole body on the lifespans between 29 and 25 °C.

Acknowledgments

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.01.099.

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