



# Methionyl-tRNA Synthetase Regulates Lifespan in *Drosophila*

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**Methionyl-tRNA synthetase (MRS) is essential for translation. MRS mutants reduce global translation, which usually increases lifespan in various genetic models. However, we found that MRS inhibited *Drosophila* reduced lifespan despite of the reduced protein synthesis. Microarray analysis with MRS inhibited *Drosophila* revealed significant changes in inflammatory and immune response genes. Especially, the expression of anti-microbial peptides (AMPs) genes was reduced. When we measured the expression levels of AMP genes during aging, those were getting increased in the control flies but reduced in MRS inhibition flies age-dependently. Interestingly, in the germ-free condition, the maximum lifespan was increased in MRS inhibition flies compared with that of the conventional condition. These findings suggest that the lifespan of MRS inhibition flies is reduced due to the down-regulated AMPs expression in *Drosophila*.**

**Keywords:** anti-microbial peptides, *Drosophila*, lifespan, methionyl-tRNA synthetase

## INTRODUCTION

Dietary restriction (DR) extends the lifespan in genetic model organisms including yeast, nematode, fruit fly, and rodents

(Liang et al., 2018; Partridge et al., 2005). Extended lifespan by DR occurs by conserved molecular mechanisms: insulin/insulin-like growth factor signaling (IIS) and target of rapamycin (TOR) pathway (Kapahi et al., 2017). IIS pathway regulates development, growth, metabolism, stress responses, fecundity, and lifespan (Broughton and Partridge, 2009; Ikeya et al., 2002). TOR pathway regulates protein translation and metabolism (Bjedov et al., 2010). In the DR condition, the activation of AMPK and Sirt is observed by suppressing IIS and TOR pathway, which contribute to lifespan extension (Fullerton and Steinberg, 2010; Tzatsos and Kandror, 2006; Xiao et al., 2011). Recently, the effect of dietary methionine restriction (MR) on lifespan received attention because MR also extends lifespan in genetic model organisms (Barcena et al., 2018). MR reduces global translation by suppressing TOR-S6K pathway and extends lifespan in worms and mammals (Barcena et al., 2018; Edwards et al., 2015). The inhibition of protein synthesis by the reduction of S6K activation is linked to IIS because TOR-S6K pathway also works as a downstream of IIS. Thus, DR and MR reduce IIS and TOR related protein synthesis, which affects lifespan extension in animals.

Aminoacyl-tRNA synthetases (ARS) are enzymes which attach a particular amino acid to its tRNA counterpart and produce aminoacyl-tRNAs for translation (Lee et al., 2004). ARS are essential enzymes for protein synthesis and cell viability (Han et al., 2012). Besides their roles in translation,

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ARS mediates diverse non-translational functions such as cell metabolism, tumorigenesis, angiogenesis, and innate immunity (Ko et al., 2000; 2001; Park et al., 2005). These non-translational functions are regulated by the multi-tRNA synthetase complex (MSC) (Han et al., 2006). Under various stress conditions, several MSC components including methionyl-tRNA synthetase (MRS) are released from the complex through post-translational modifications (Lee et al., 2016). MRS is a key factor in translation initiation and also play a role in the biogenesis of rRNA, translational fidelity under oxidative stress, and oncogenic transformation in the overexpression condition (Ko et al., 2000; Netzer et al., 2009). Although MRS has evolutionary conserved and significant in translation control, the physiological function of MRS at the organism level is not well studied.

In *Drosophila*, anti-microbial peptides (AMPs) show defense functions against infections (Lemaitre et al., 1997). Expression of AMPs genes is regulated by NF- $\kappa$ B family transcription factors including Relish (Fabian et al., 2018). In addition, subsets of AMPs are directly regulated by another transcription factor, dFOXO (Becker et al., 2010). The elevated innate immune system is a common feature of aged animals including *Drosophila* (Zerofsky et al., 2005). Aged flies also show increased transcription levels of AMPs genes (Zerofsky et al., 2005). However, how mRNA expressions of AMPs genes are regulated in aged flies are largely unknown.

Here, we inhibited the expression of the *MRS* gene using the chemically-induced conditional knock-down system to investigate the lifespan of *MRS* inhibited *Drosophila*. When the expression of the *MRS* gene is inhibited, the lifespan was reduced on the contrary to the expectation. We found that the reduced lifespan of *MRS* inhibition flies is due to down-regulated AMPs expression.

## MATERIALS AND METHODS

### *Drosophila* culture and stocks

Flies were maintained at 25°C on standard cornmeal, yeast, sugar, agar medium (standard medium). *w<sup>1118</sup>*, *Actin<sup>GS</sup>-Gal4*, and *UAS-MRS RNAi* were obtained from the Bloomington Stock Center (USA). For the activation of gene switch system, 20  $\mu$ g/ml mifepristone (RU486; Sigma, USA) was mixed in the standard medium.

### Generation of germ-free *Drosophila melanogaster* and fly husbandry

Germ-free flies were generated by bleaching the embryos. Embryos of *Actin<sup>GS</sup>-Gal4* and *UAS-MRS RNAi* were collected for 12 h and then dechorionated for 50 s in 5% sodium hypochlorite solution (Wako Chemicals, USA), rinsed for 50 s in 70% ethanol, and washed for 1 min in sterile distilled water. Sterile embryos were transferred into sterile standard cornmeal-sugar-yeast (CSY) food bottles on a clean bench. Eggs in a germ-free condition were passed through repeated generations and became second-generation flies. All germ-free flies were maintained on a clean bench and were transferred to fresh food every two days. The germ-free conditions were confirmed by plating fly homogenate on plate count agar (PCA; Neogen Corporation, USA), and by 16S rRNA

gene PCR with a bacterial 16S rRNA universal primers (27F and 1492R) provided by MacroGen. CSY media were used during culture and rearing of the flies. To produce the sterile CSY diet, CSY medium (5.2% cornmeal, 11% sugar, 2.5% instant yeast, 0.5% propionic acid, 0.04% methyl-4-hydroxybenzoate, 1% agar) was autoclaved at 120°C for 20 min, and all bottles containing food were exposed to UV light for 20 min on a clean bench.

### Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

From the wholebody of 10 adult flies, total RNA was isolated with the easy-BLUE reagent (iNtRON Biotechnology, Korea). After treating the RNA samples with RNase-free DNase I (Takara, Japan), cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen, USA). Quantitative RT-PCR analysis was performed with the CFX connect (BioRad, USA) using the Syber Green PCR Core reagents (Toyobo, Japan). Each experiment was performed at least three times and the comparative cycle threshold was used to present a fold change for each specific mRNA after normalizing to *rp49* levels. The primers used in the qPCR analyses are listed in Supplementary Table S1.

### Microarray analysis

For each RNA, the synthesis of target cRNA probes and hybridization were performed using Agilent's Low Input Quick-Amp Labeling Kit (Agilent Technologies, USA) according to the manufacturer's instructions. The hybridization images were analyzed by Agilent DNA microarray Scanner (Agilent Technologies) and the data quantification was performed using Agilent Feature Extraction software 10.7 (Agilent Technologies). Functional annotation of genes was performed according to Gene Ontology TM Consortium (<http://www.geneontology.org/index.html>) by GeneSpring GX 7.3.1.

### SUnSET assay

Surface sensing of translation (SUnSET) was performed (Schmidt et al., 2009) on the control (*Actin<sup>GS</sup> > MRS RNAi*, RU-) and *MRS* inhibition flies (*Actin<sup>GS</sup> > MRS RNAi*, RU+). Whole body of each genotype flies were incubated in Shields and Sang M3 insect medium (cat. #S8398; Sigma) containing 2  $\mu$ g/ml puromycin (cat. #P8833; Sigma) for 3 h at 25°C. After puromycin incubation, Western blotting was performed to confirm the levels of puromycin incorporation with actin as a loading control. Mouse anti-Puromycin primary antibody (cat. #MABE343; Millipore, USA) was used at 1:1,000 dilution. Each sample was loaded in parallel on two gels/membranes so that actin band could be distinguished from puromycin containing peptides.

### Western blot

Total protein was isolated from adult bodies of flies with the PROPREP protein extraction buffer (iNtRON Biotechnology) or RIPA buffer (Cell Signaling, USA). The fly proteins (30  $\mu$ g) was subjected to SDS-PAGE and transferred to PVDF membrane (Millipore). The membranes was blocked for 2 h in TBST buffer containing 5% non-fat milk and incubated them overnight at 4°C in TBST buffer containing 5% bovine serum

albumin with primary antibodies. After the incubation with secondary antibodies and subsequent washes, images of the membranes were digitized using the Fluorchem E (Protein Simple, USA). Then, target proteins versus their internal loading control band intensities were quantified with the Image J software. Following primary and secondary antibodies were used: anti-phospho-S6K (1:1,000; Cell Signaling), anti-puromycin (1:1,000; Millipore), anti-GFP (1:2,000; Santa Cruz Biotechnology, USA), anti- $\beta$ -actin for *Drosophila* (1:1,000; Developmental Studies Hybridoma Bank [DSHB], USA), Goat anti-rabbit IgG (1:3,000; Cell Signaling), and Goat anti-mouse IgG (1:3,000; Cell Signaling).

### Lifespan assay

Lifespan was measured in adult flies kept in standard medium and RU486 medium. Newly eclosed male adult flies from each genotype were collected and assigned 20 flies per vial in normal fly food and RU486 medium. Flies were transferred and dead flies were recorded every 2 or 3 days.

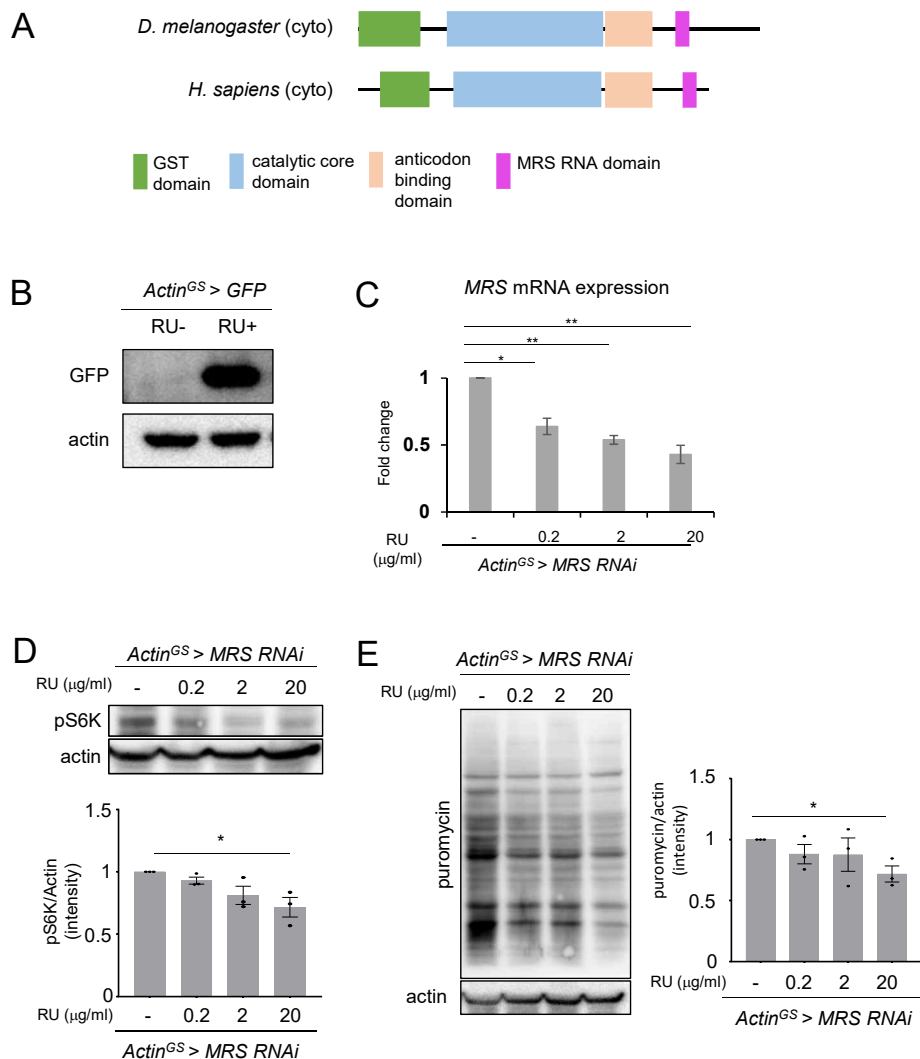
### Statistics

All experiments were repeated at least three times and the data were shown as the mean and error bar ( $\pm$ SEM) using Prism7.02 (GraphPad Software, USA). Student's *t*-test was used to compare each group and  $P < 0.05$  was considered as statistically significant. For lifespan analyses, log-rank tests were performed using the online survival analysis tool OASIS2 (Han et al., 2016).

## RESULTS

### Protein translation is reduced in MRS inhibited *Drosophila*

MRS is a key regulator of translation process and an evolutionary conserved enzyme from insect to human. *Drosophila* MRS is an evolutionary conserved protein with GST domain, catalytic domain, anti-codon binding domain, and MRS RNA domain like those in the human MRS (Fig. 1A). To study the *in vivo* function of MRS, we used Gene Switch (GS) Gal4/UAS system. To test this system, *UAS-GFP* flies were crossed with *Actin<sup>GS</sup>-Gal4* (*Actin<sup>GS</sup> > GFP*) without RU486 chemical



**Fig. 1. Evolutionary conserved domains of *Drosophila* MRS, the efficacy of GeneSwitch system, and protein translation in MRS inhibition flies.** (A) Evolutionary conserved domains between human MRS and *Drosophila* MRS. (B) GFP protein level was highly elevated in *Actin<sup>GS</sup> > GFP* flies with RU486 20  $\mu$ g/ml (RU+). (C) MRS mRNA expression was getting reduced in *Actin<sup>GS</sup> > MRS RNAi* flies RU dose dependently. (D) Protein translation level marked by the phosphorylated S6K protein was reduced in MRS inhibition flies RU dose dependently. (E) Global translation marked by puromycin incorporation was reduced in MRS inhibition flies RU dose dependently. RU 0.2, RU 2, and RU 20: *Actin<sup>GS</sup> > MRS RNAi* with RU486 0.2  $\mu$ g/ml, 2  $\mu$ g/ml, 20  $\mu$ g/ml, respectively. Data are presented as mean  $\pm$  SEM; \* $P < 0.05$  and \*\* $P < 0.01$ .

inducer (RU-) and with RU486 (RU+). In the RU+ condition, GFP was strongly expressed (Fig. 1B). To inhibit MRS expression in the whole body of adult flies, *UAS-MRS RNAi* flies were crossed with *Actin<sup>GS</sup>-Gal4* (*Actin<sup>GS</sup> > MRS RNAi*) and RU486 was fed in the adult *Actin<sup>GS</sup> > MRS RNAi* flies. The expression level of MRS mRNA in adult *Actin<sup>GS</sup> > MRS RNAi* flies was RU dose-dependently decreased compared with RU-control (Fig. 1C).

We examined whether MRS inhibition flies (*Actin<sup>GS</sup> > MRS RNAi*, RU+) reduces protein translation. *Actin<sup>GS</sup> > MRS RNAi* flies with RU 20 µg/ml significantly reduced the pS6K level, a protein translation marker, compared with that of the RU-control (Fig. 1D). In the SUnSET assay, a global translational assay, puromycin incorporation level was reduced in *Actin<sup>GS</sup> > MRS RNAi* flies with RU 20 µg/ml compared with that of the RU- control (Fig. 1E). These results indicate that *Actin<sup>GS</sup> > MRS RNAi* flies with RU 20 µg/ml significantly reduced protein synthesis as expected.

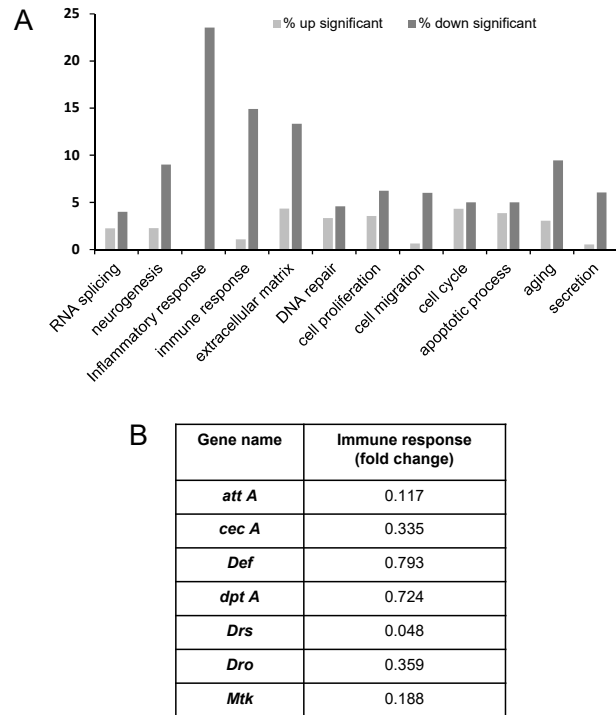
### Lifespan is reduced in MRS inhibited *Drosophila*

Next, we measured lifespan in *Actin<sup>GS</sup> > MRS RNAi* flies. Genetic or chemical inhibition of protein translation extends lifespan from insect to mammals (Bjedov et al., 2010; Kapahi et al., 2004). However, MRS inhibition by *Actin<sup>GS</sup> > MRS RNAi* with RU+ reduced median and maximum lifespan dose-dependently compared with those of the RU- control (Fig. 2). These results suggest that MRS inhibition regulates lifespan by factors other than protein translation.

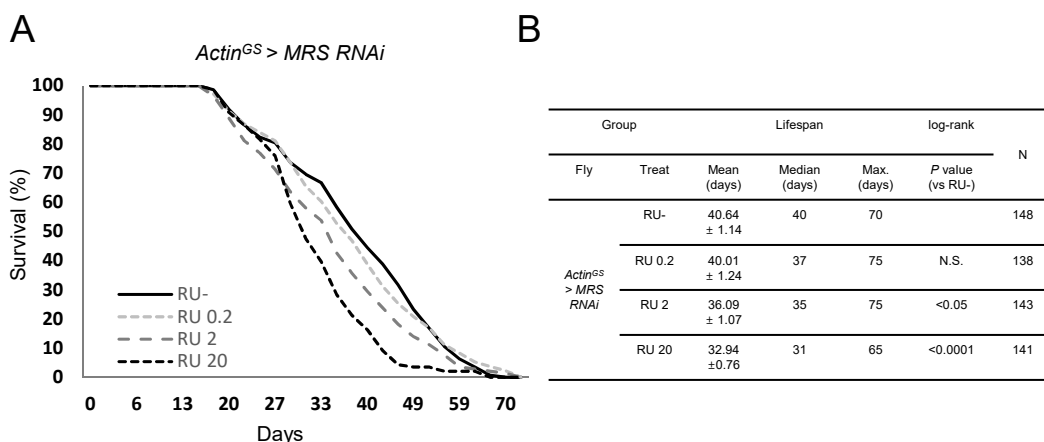
### Inflammation and immune response genes are downregulated in MRS inhibited *Drosophila*

To find factors regulating the lifespan of *Actin<sup>GS</sup> > MRS RNAi* flies at the genome level, we performed mRNA microarray analysis using 30 days old *Actin<sup>GS</sup> > MRS RNAi* flies. Interestingly, gene ontology classification showed that significantly downregulated genes were in inflammatory and immune response genes (Fig. 3A). AMPs are involved in inflammatory and immune response (Hoffmann, 2003; Stoven et al., 2000). In 30 days old *Actin<sup>GS</sup> > MRS RNAi* flies, the expression

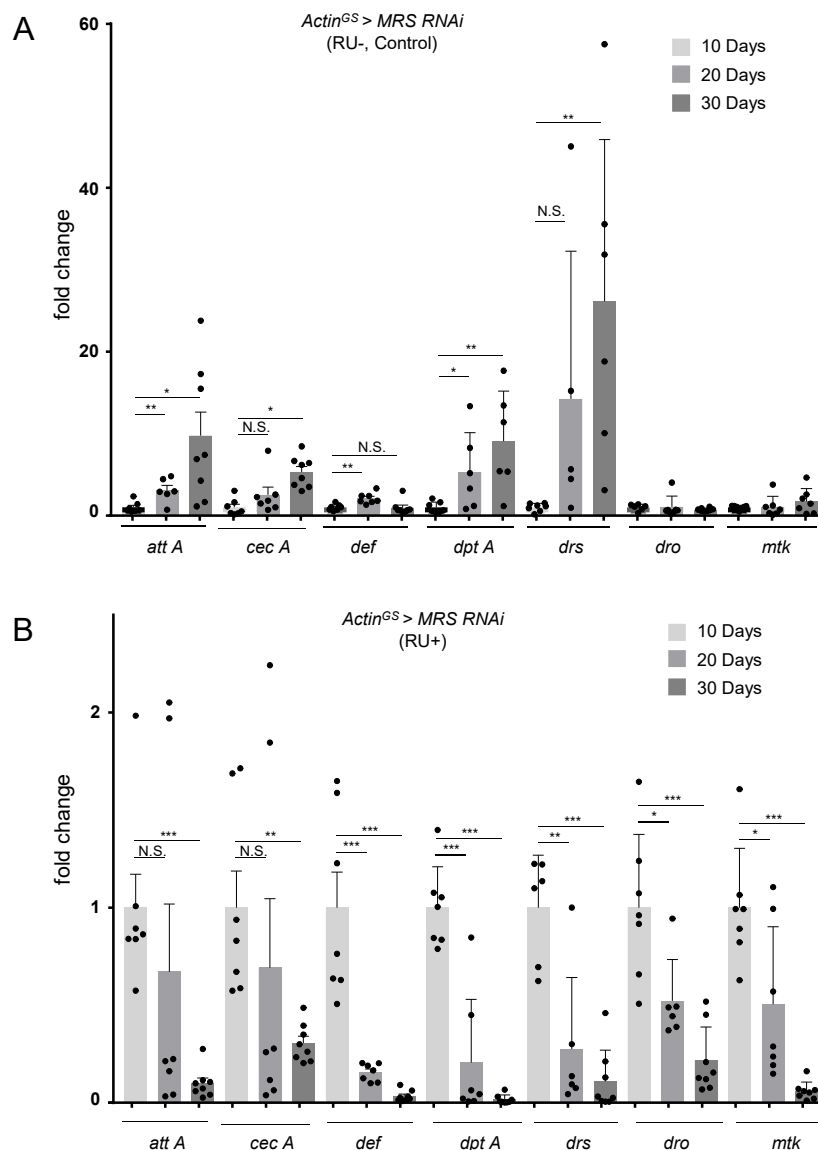
levels of AMPs (*attacin A* [*att A*], *cecropin A* [*cec A*], *defensin* [*Def*], *diphtericin A* [*Dpt A*], *Drosocin* [*Drs*], *Drosomycin* [*Dro*], and *Metchnikowin* [*MTK*]) were significantly reduced (Fig. 3B).



**Fig. 3. MRS regulates inflammatory and immune response genes.** (A) Gene ontology graph from the microarray analysis with 30 days old MRS inhibition flies showed that the genes involved in inflammatory and immune responses were changed significantly. (B) Microarray analysis showed that the mRNA expression of AMPs (*attA*, *cecA*, *Def*, *DptA*, *Drs*, *Dro*, and *MTK*) were down-regulated in 30 days old MRS inhibition flies.



**Fig. 2. Lifespan in MRS inhibition flies.** (A) The lifespan of MRS inhibition flies were reduced RU dose dependently. (B) Statistic table of lifespan analysis. RU 0.2, RU 2, and RU 20: *Actin<sup>GS</sup> > MRS RNAi* with RU486 0.2 µg/ml, 2 µg/ml, 20 µg/ml.



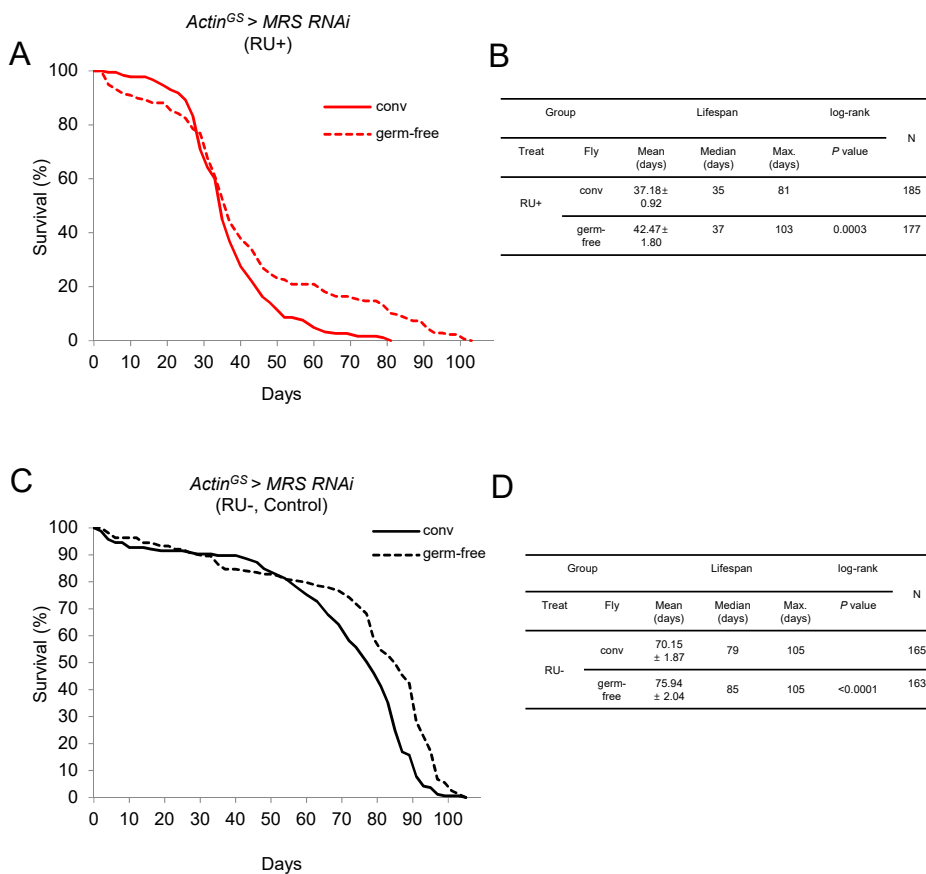
**Fig. 4. Expression of AMPs is reduced by aging in MRS inhibition flies.** (A) In the control (*Actin<sup>GS</sup> > MRS RNAi*, RU-), the mRNA expression levels of AMPs (*attA*, *cecA*, *Def*, *DptA*, *Drs*, *Dro*, and *MTK*) were increased during aging. (B) In MRS inhibition flies (*Actin<sup>GS</sup> > MRS RNAi*, RU+), the mRNA expression levels of AMPs (*attA*, *cecA*, *Def*, *DptA*, *Drs*, *Dro*, and *MTK*) were reduced by aging. RU+, *Actin<sup>GS</sup> > MRS RNAi* with RU486 20  $\mu$ g/ml. Data are presented as mean  $\pm$  SEM; \**P* < 0.01, \*\**P* < 0.01, and \*\*\**P* < 0.001. N.S., statistically not significant.

#### Expressions of AMPs is reduced in aged MRS inhibited *Drosophila*

In the previous report, the expression levels of AMPs were increased age-dependently (Zerofsky et al., 2005). We confirmed it in the *Actin<sup>GS</sup> > MRS RNAi* RU- control and *w<sup>1118</sup>* flies using the expression levels of *att A*, *cec A*, *Def*, *Dpt A*, *Drs*, *Dro*, and *MTK* genes (Fig. 4A). However, in MRS inhibition flies by *Actin<sup>GS</sup> > MRS RNAi* RU+, the expression levels of *att A*, *cec A*, *Def*, *Dpt A*, *Drs*, *Dro*, and *MTK* genes were decreased by aging (Fig. 4B). These data suggest that the reduced lifespan of MRS inhibition flies may relate to the lack of innate immune response by the decreased AMPs expression.

#### The lifespan of MRS inhibited *Drosophila* is increased in the germ-free condition

To test whether the reduced lifespan of MRS inhibition flies may relate to the lack of innate immune response, the lifespan of MRS inhibition flies was tested in the germ-free condition. Surprisingly, the maximum lifespan of MRS inhibition flies was increased significantly in the germ-free condition compared with the conventional condition (Figs. 5A and 5B). In the control *Actin<sup>GS</sup> > MRS RNAi* RU- flies, the maximum lifespan was not changed in the germ-free condition compared with the conventional condition (Figs. 5C and 5D).



**Fig. 5. The lifespan of MRS inhibition flies is increased in the germ-free condition.** (A) The maximum lifespan of MRS inhibition flies was increased in the germ-free condition (*Actin<sup>GS</sup> > MRS RNAi*, RU+; red dotted line) compared with that of the conventional condition (*Actin<sup>GS</sup> > MRS RNAi*, RU+; red solid line). (B) Statistic table of lifespan analysis of Fig. 5A. (C) The maximum lifespan of control flies did not increase in the germ-free condition (*Actin<sup>GS</sup> > MRS RNAi*, RU-, dotted line) compared with that of the conventional condition (*Actin<sup>GS</sup> > MRS RNAi*, RU-, solid line). (D) Table of lifespan analysis of Fig. 5C. RU-, *Actin<sup>GS</sup> > MRS RNAi* without RU486.

## DISCUSSION

It has been known that DR including MR extends the lifespan of animals, which are generally controlled by Insulin signaling and TOR pathway (Kapahi et al., 2017; Min et al., 2008). In this study, we showed an unexpected finding that lifespan was reduced in MRS inhibition flies although the phosphorylation of S6k and global translation level were decreased. We suggest that the reduced lifespan by MRS inhibition is caused by the downregulation of immune response-relating genes. Previous studies reported that aged flies increase AMPs expressions to overcome their immune deficient responses (Zerofsky et al., 2005). The age-dependent diminution of immune response, called immune senescence, is a general phenomenon of aging process (Kuk et al., 2019; Min and Tatar, 2018). Aging is accompanied by chronic inflammation, deterioration of protective gut epithelial barriers, increased pathogen susceptibility, and deregulation of gut microbiota dysbiosis (Min and Tatar, 2018). Consistent with our data of age-dependently elevated expression of AMPs (Fig. 4), the previous report shows that *relish* and AMPs expression were increased during aging and overexpression of *relish* and individual AMPs shortened lifespan (Badinloo et al., 2018). MRS inhibition flies seem to mimic immune senescence in the young stage, which results in reduced lifespan.

The microarray data with aged MRS inhibition flies indicate that the expression levels of genes belonging to extracellular

matrix, DNA repair, cell proliferation, cell migration, apoptosis, and aging were also changed (Fig. 3A). Deprivation of amino acids, especially the essential amino acids deprivation, exhibits severe loss of cell viability and increased cell death by apoptosis compared with non-essential amino acids deprivation (Simpson et al., 1998). For example, starvation of lysine or histidine, one of the essential amino acids, induces apoptotic cell death through LC3 activation, an autophagy marker. This cell death induces high level of oxidative stress (Eisler et al., 2004). MRS inhibition flies may function as similar to other essential amino acid deprivations in addition to the down-regulated AMPs expression. Therefore, MRS inhibition flies may also induce cell death-mediated oxidative stress, which affects DNA repair, cell proliferation, cell migration, apoptosis, and aging processes.

To address the relationship between MRS and immune responses in lifespan, we tested the lifespan of MRS inhibition flies in the germ-free condition to exclude the effect of commensal bacteria-induced immune system activation. Lifespan studies of wild-type flies in the germ-free condition is controversial. One study showed that the lifespan of wild-type flies in the germ-free and antibiotics medium is reduced than that of the control flies (Brummel et al., 2004), whereas another study reported that the lifespan of wild-type flies in the germ-free condition is extended compared with the conventional condition (Petkau et al., 2014) like our experimental data (Fig. 5B). When we tested the lifespan of MRS inhibition flies

in the germ-free condition, the maximum lifespan of *MRS* inhibition flies increased compared with that of the control (Figs. 5A and 5B). In the germ-free condition, the increased lifespan of *MRS* inhibition mutants may be due to the reduced protein translation.

Recently, *MRS* has emerged as a target to develop therapeutics for malaria. Malaria is usually caused by *Plasmodium falciparum* infection. *MRS* of *P. falciparum* is a new drug candidate. Several malaria *MRS* inhibitors (REP3123 and REP8839), previously known as bacterial inhibitors, are found by in silico screening (Khan, 2016). Taken together, we suggest a new connection between lifespan and immune response through *MRS*.

Note: Supplementary information is available on the Molecules and Cells website ([www.molcells.org](http://www.molcells.org)).

## Disclosure

The authors have no potential conflicts of interest to disclose.

## ACKNOWLEDGMENTS

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