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# A novel *Drosophila* mitochondrial carrier protein acts as a $Mg^{2+}$ exporter in fine-tuning mitochondrial $Mg^{2+}$ homeostasis

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ABSTRACT

The homeostasis of magnesium ($Mg^{2+}$), an abundant divalent cation indispensable for many biological processes including mitochondrial functions, is underexplored. In yeast, the mitochondrial $Mg^{2+}$ homeostasis is accurately controlled through the combined effects of importers, Mrs2 and Lpe10, and an exporter, Mme1. However, little is known about this $Mg^{2+}$ homeostatic process in multicellular organisms. Here, we identified the first mitochondrial $Mg^{2+}$ transporter in *Drosophila*, the orthologue of yeast Mme1, dMme1, by homologous comparison and functional complementation. dMme1 can mediate the exportation of mitochondrial $Mg^{2+}$ when heterologously expressed in yeast. Altering the expression of dMme1, although only resulting in about a 10% change in mitochondrial $Mg^{2+}$ levels in either direction, led to a significant survival reduction in *Drosophila*. Furthermore, the reduced survival resulting from dMme1 expression changes could be completely rescued by feeding the *dMME1*-RNAi flies $Mg^{2+}$-restricted food or the *dMME1*-over-expressing flies the $Mg^{2+}$-supplemented diet. Our studies therefore identified the first *Drosophila* mitochondrial $Mg^{2+}$ exporter, which is involved in the precise control of mitochondrial $Mg^{2+}$ homeostasis to ensure an optimal state for survival.



## 1. Introduction

Magnesium ($Mg^{2+}$), a major intracellular divalent cation in living cells, functions as a co-factor for hundreds of enzymes [1]. It is of critical importance for various metabolic reactions, including energy production, protein and nucleic acid synthesis, cell cycle regulation and ion metabolism [2]. $Mg^{2+}$ is closely related to many important human diseases such as type 2 diabetes mellitus, hypertension and cardiovascular diseases [3–5].

Despite the great importance of this cation, our understanding of how cells regulate the transportation and homeostasis of $Mg^{2+}$ remains quite preliminary. The TRP (Transient Receptor Potential) superfamily represents one type of cation channels critical for $Mg^{2+}$ homeostasis [6–8]. In fruit flies or *Drosophila melanogaster*, one member of this family, the TRPM protein, was found to be essential for viability and for control of $Mg^{2+}$ levels in the hemolymph [9]. The magnesium transporter subtype 1 (MagT1) is a newly discovered and evolutionarily conserved magnesium transporter [10]. A previous study has shown that the *Drosophila* homolog of mammalian MagT1 (dMagT1) is a $Mg^{2+}$ selective transporter with channel-like properties, and is up-regulated by PKC activation [11].

Mitochondria are an important organelle for $Mg^{2+}$ homeostasis [12]. In yeast, the mitochondrial inner membrane proteins – Mrs2/Lpe10 – function in transporting $Mg^{2+}$ into the mitochondria, which serves as a major intracellular $Mg^{2+}$ pool [13–18]. Deletion of either of these two genes leads to decreased mitochondrial $Mg^{2+}$ levels, growth inhibition on non-fermentative medium, and group II RNA splicing defects [13,14]. Recently, our lab identified, a novel yeast mitochondrial $Mg^{2+}$ exporter, Mme1, for its ability to rescue the phenotypes of *mrs2Δ* and *lpe10Δ* mutants when deleted [19]. Conversely, yeast strains over-expressing *MME1* displayed similar phenotypes as the *mrs2Δ* and *lpe10Δ* mutants [19]. Both in vivo studies and in vitro transportation assays in proteoliposomes support the claim that Mme1 is responsible for exporting $Mg^{2+}$ out of the mitochondria and into the cytosol [19]. Mme1 works together with $Mg^{2+}$ importers, Mrs2/Lpe10, to enforce a two-level control of mitochondrial $Mg^{2+}$ homeostasis to ensure a delicate balance between mitochondrial and cytosolic $Mg^{2+}$ levels. Any disturbance of this balance would cause a detrimental effect to the yeast cell under $Mg^{2+}$-deficient conditions [19].

Our knowledge of mitochondrial $Mg^{2+}$ homeostasis remains lacking for multicellular organisms. No Mme1 or mitochondrial $Mg^{2+}$ exporter has been reported so far in any higher organism. In this study, we used *D. melanogaster*, a powerful and genetically well tractable model organism, to isolate a Mme1 functional homolog. By homologous comparison and functional complementation in yeast, we identified a novel

*Abbreviation:* $Ca^{2+}$, Calcium; ICP-MS, Inductively coupled plasma mass spectrometry; IPTG, Isopropyl-β-D-thiogalactopyranoside; $Mg^{2+}$, Magnesium; NSD, Normal synthetic diet; RT-PCR, Reverse transcription polymerase chain reactions; $Zn^{2+}$, Zinc.

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candidate — *CG3476*, as a possible *Drosophila* orthologue of yeast Mme1. Subsequent characterization showed that *CG3476* indeed functions as a *Drosophila* mitochondrial $Mg^{2+}$ exporter, and therefore we renamed it dMme1. Modulating the expression of *dMME1* causes the mitochondrial $Mg^{2+}$ level to be modestly altered, resulting in significant survival reduction. Therefore, for optimal well-being of a multicellular organism such as *D. melanogaster*, the intricate mitochondrial $Mg^{2+}$ homeostasis is presumably also maintained by both the exporter dMme1 and importers, whose identity remain unknown in the fly.

## 2. Results

### 2.1. A Mme1 candidate in D. melanogaster is isolated by sequence comparison and yeast functional complementation

Our previous work reported a novel mitochondrial $Mg^{2+}$ exporter, Mme1, in yeast. To identify the potential Mme1 orthologue in *Drosophila*, we used the NCBI Blast tool (Blast.ncbi.nlm.nih.gov) to compare *Drosophila* genome using Mme1 as the query. Five *Drosophila* genes (*CG4743*, *CG32103*, *CG3476*, *CG4995*, and *colt*) with highest similarities to yeast *MME1* were expressed in *mme1Δ mrs2Δ* double mutant strains to test whether any of these genes could substitute the function of *MME1*, for example, restore the inability of the yeast to grow on non-fermentable media, in which glycerol is the sole carbon source. We did not use a *mme1Δ* single mutation strain because *MME1* deletion by itself has very minor, if at all, observable growth phenotypes [19]. We reasoned that deletion of yeast *MME1* in *mrs2Δ* mutants can suppress the growth defect of *mrs2Δ* mutants on non-fermentable media, and if a *Drosophila* gene functions similarly as *MME1*, it might be able to compensate the *MME1* mutation and help reinstate the growth defect of *mme1Δ mrs2Δ* double mutants [19].

The five candidate genes were cloned into a GPD promoter-driven centromeric vector, to achieve an appropriate level of expression. Spotting assay results are shown in Fig. 1. Candidates sharing different sequence similarities are arranged sequentially with the highest similarity at the top and the lowest at the bottom. It is obvious that only the expression of *CG3476* exhibited a similar growth inhibition effect as yeast *MME1*, indicating that *CG3476* might be an orthologue of yeast *MME1* (Fig. 1). Interestingly, *Drosophila CG3476*, which displays about 30% identity and 45% similarity to yeast *MME1*, is not the one which shows the highest similarity to yeast *MME1*.

### 2.2. Heterologous expression of CG3476 in wild-type yeast leads to significant mitochondrial $Mg^{2+}$ decrease and growth defects

Mitochondrial $Mg^{2+}$ is critical for the growth of yeast on non-fermentable glycerol media and the RNA splicing of group II introns [13,14,20]. To further characterize whether CG3476 is involved in regulating mitochondrial $Mg^{2+}$, we tested the effects of heterologous expression of *CG3476* in wild-type yeast. When *CG3476* was strongly expressed with pTEF-425, a multiple-copied yeast expression vector with a *TEF1* promoter, significant growth defects and group II RNA splicing defects occurred with growth on respiratory medium (Fig. 2A and B). We then sought to test whether the mitochondrial $Mg^{2+}$ levels were affected directly by *CG3476*-expression or not. The contents of $Mg^{2+}$ and $Zn^{2+}$ in mitochondria were measured by inductively coupled plasma mass spectrometry (ICP-MS). As shown in Fig. 2C, expression of *CG3476* resulted in an appreciably lower level of mitochondrial $Mg^{2+}$ content, while it had little effects on the level of $Zn^{2+}$. These phenotypes due to *CG3476* over-expression are quite similar with those of yeast *MME1* over-expression that we previously reported [19], strongly suggesting that *CG3476* encodes a *Drosophila* mitochondrial $Mg^{2+}$ exporter and is hereafter named *dMME1*.

### 2.3. Heterologous expression of dMME1 can enhance $Mg^{2+}$ exportation in E. coli

Based on sequence similarity, dMme1 was predicted to belong to the mitochondrial carrier family. Mitochondria are thought to originate from proteobacteria that were taken up by some sort of nucleated cell, and in some aspects mimic bacterial cells such as *E. coli*. To obtain greater evidence to support the hypothesis that dMme1 functions as a mitochondrial $Mg^{2+}$ exporter, $Mg^{2+}$ efflux activity of dMme1 was examined in a semi-in-vitro biosystem — the *E. coli*.

It was initially difficult to significantly express dMme1 in *E. coli*. Nonetheless, a low expression of dMme1 was finally achieved by using the pET15b plasmid vector with an N-terminal HIS-tag and *E. coli* host strain BL21 (DE3). Results of confocal microscopy showed that the heterologously expressed dMme1 protein was localized in the

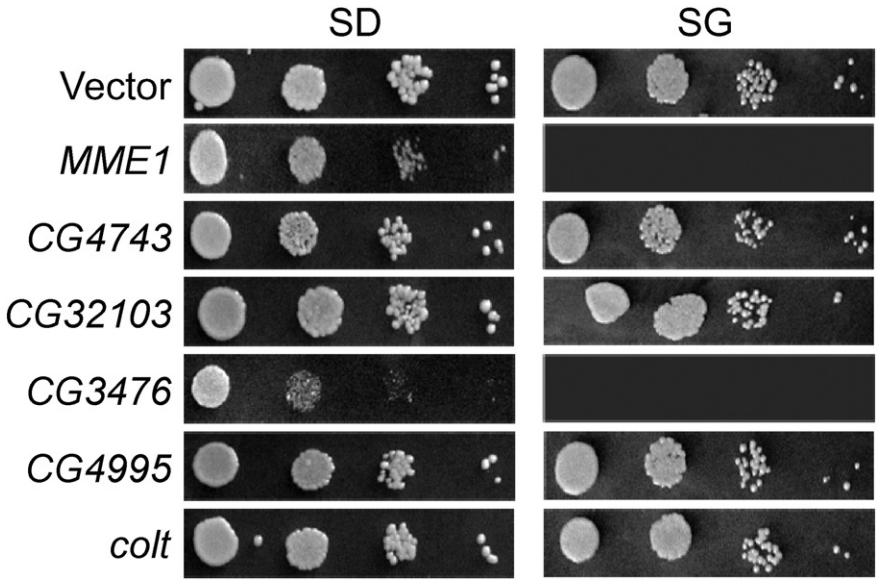


**Fig. 1.** Identification of *Drosophila* Mme1 orthologue. CG3476 was identified as the *Drosophila* orthologue of yeast Mme1 by functional complementation. Candidates of Mme1 homolog in *Drosophila* were expressed in *mme1Δ mrs2Δ* yeast to test their abilities to suppress growth on synthetic glycerol (SG) medium, in which the respiration function of mitochondrial is needed. Synthetic glucose (SD) medium serves as spotting control. Glycerol and D-glucose is the carbon source in SG and SD medium respectively. Five of the *Drosophila* candidate genes with the highest homology to yeast *MME1* were cloned into pGPD-413 vector, which is a centromeric yeast expression vector with the *GPD* promoter. Yeast *MME1* served as the positive control.

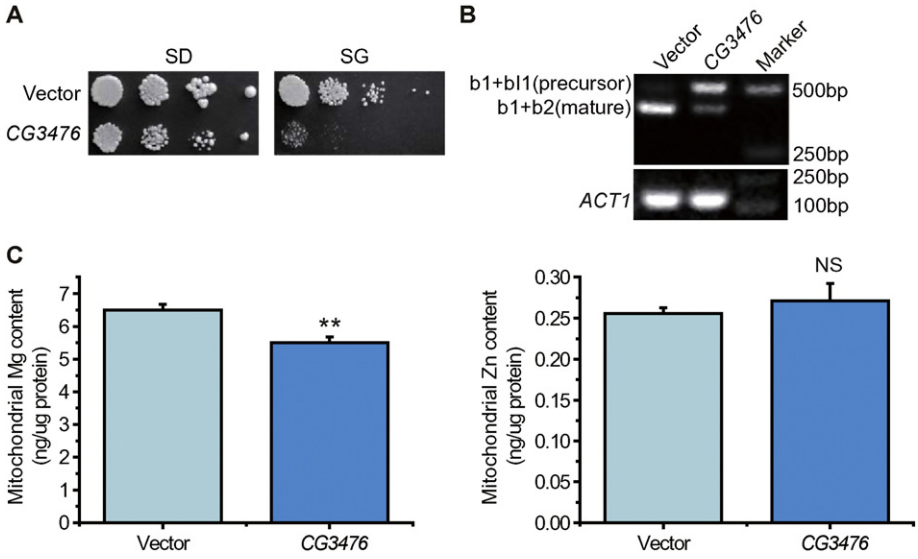


**Fig. 2.** Phenotypes of expressing *CG3476* in wild-type yeast. (A) Strong expression of *CG3476* in wild-type yeast cells conferred a growth inhibition on the glycerol medium (SG). To achieve a higher *CG3476* expression level, *CG3476* was cloned into a pTEF-425 vector which is a multiple-copied yeast expression vector with a *TEF1* promoter. (B) Effect of the expression of *CG3476* on yeast mitochondrial group II intron RNA splicing. RT-PCR assays were performed with a mixture of three oligonucleotide primers matching two different exons (b1 and b2) and one intron (bI1) of *COB*. The PCR product amplified by b1 and b2 is the mature exon-exon junction of *COB*, while b1 and bI1 is the immature exon-intron precursor. PCR product of *ACT1* was used as the loading control. (C) Effect of the expression of *CG3476* on the mitochondrial $Mg^{2+}$ contents in wild-type yeast cells. $Zn^{2+}$ serves as a negative control. Values are presented as mean ± S.D.; n ≥ 3. NS stands for no significance.

peripheral area (plasma membrane) of *E. coli*, coinciding with the presumed localization of dMme1 in the mitochondrial membrane of eukaryotic cells (Fig. 3A). We next asked whether the expression of dMme1 in such a foreign system could facilitate cellular $Mg^{2+}$ efflux. Further analysis of cellular $Mg^{2+}$ content indicated that expression of dMme1 indeed significantly and specifically reduced the $Mg^{2+}$ levels of the cells (Fig. 3B). These semi-in-vitro results suggest that dMme1 is necessary and sufficient in mediating mitochondrial $Mg^{2+}$ efflux.

### *2.4. dMme1 knock-down leads to increased mitochondrial $Mg^{2+}$ contents and shortened life-spans in Drosophila*

When expressed in CHO cells, dMme1-EGFP co-localized perfectly with the mitochondrial membrane (marked by Tom22-mCherry), but not with ER-localized RFP or Golgi-localized RFP, suggesting that dMme1 is indeed a mitochondrial protein (Fig. 4A). When dMme1-EGFP fusion protein was expressed in *mme1Δ mrs2Δ* double mutant

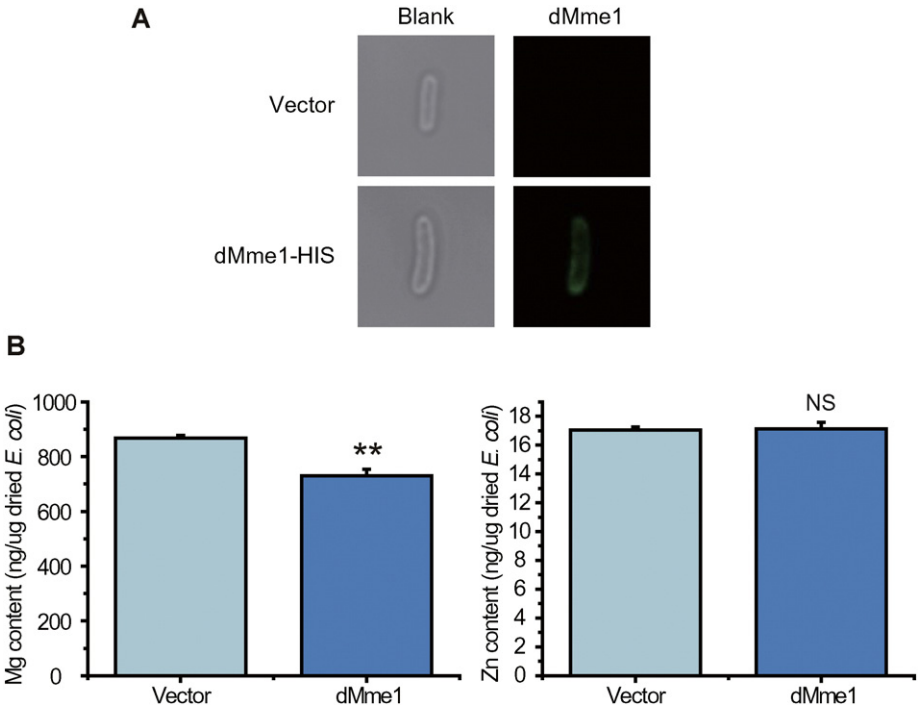


**Fig. 3.** dMme1 can mediate $Mg^{2+}$ efflux in *E. coli*. (A) Plasma-membrane-localization of dMme1 in *E. coli*. Immunofluorescence assays were carried out in BL21 (DE3) *E. coli* cells transfected with a HIS-tagged dMme1. (B) Expression of dMme1 in *E. coli* decreased $Mg^{2+}$ concentration within the cells. The contents of $Mg^{2+}$ and $Zn^{2+}$ in *E. coli* cells were measured by ICP-MS and normalized by the gross dry weight of *E. coli* cells. Values are presented as mean ± S.D.; n ≥ 3. NS stands for no significance.

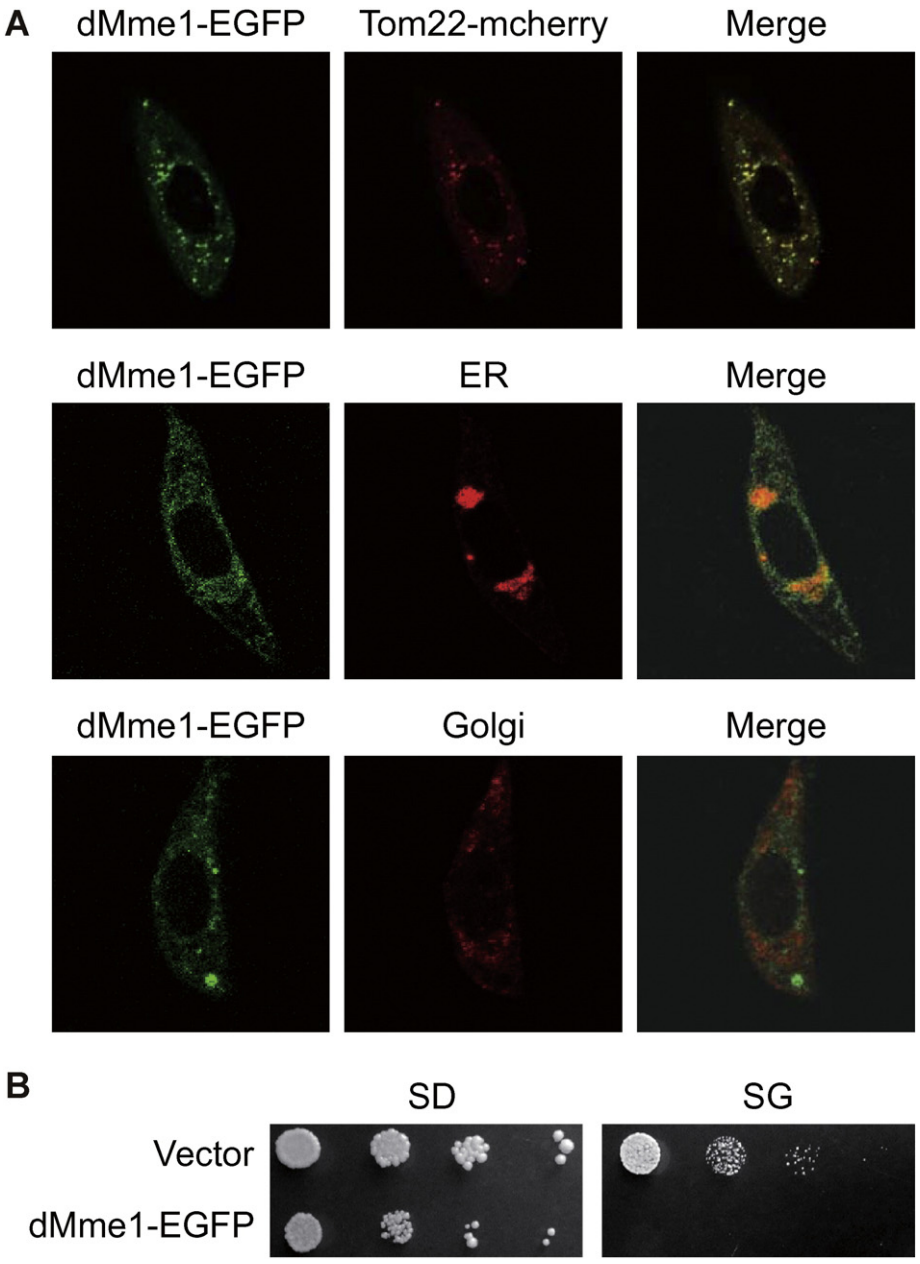


**Fig. 4.** Mitochondrial localization of dMme1 in CHO cells. (A) dMme1-EGFP protein colocalizes with mitochondrial membrane protein, Tom22-mCherry, but not with ER-localized RFP or Golgi-localized RFP. (B) Expression of dMme1-EGFP in *mme1Δ mrs2Δ* double mutant strain could suppress the growth on glycerol medium (SG), suggesting that dMme1-EGFP used to determine the mitochondrial membrane localization of dMme1 is functional.

yeast strains, the growth defect on glycerol medium was similar to the wild-type dMme1 (*CG3476*) growth defect in Fig. 1 (Fig. 4B). This suggests that the dMme1-EGFP fusion protein used for localization is functional.

We next studied the *in vivo* function of dMme1. In order to achieve universal *dMME1* silencing, *dMME1*-RNAi flies were crossed with the Gal4 driver flies ubiquitously expressing Gal4 under the regulatory sequences of *actin* (*Actin-GAL4*). As revealed by the results of reverse transcription polymerase chain reactions (RT-PCR), the abundance of *dMME1* mRNA was significantly reduced in the RNAi fly line (about 40% of control) (Fig. 5A). We then examined whether the contents of *Drosophila* mitochondrial $Mg^{2+}$ would be affected by *dMME1* knockdown. As expected, the *dMME1*-RNAi flies displayed increased $Mg^{2+}$ accumulation in the mitochondria — about 13% higher than the control. Correspondingly, there was a decrease of post-mitochondrial $Mg^{2+}$ contents in *dMME1*-RNAi flies. As a control, the levels of $Zn^{2+}$ were not significantly altered (Fig. 5B). It seems that dMme1 is involved in moving $Mg^{2+}$ out from mitochondria to the cytosol.

As $Ca^{2+}$ and $Mg^{2+}$ homeostasis could be tightly linked [21,22], we wondered whether $Ca^{2+}$ would be affected by changes of dMme1 expression. Mitochondrial and post-mitochondrial $Ca^{2+}$ levels were not significantly altered in *Drosophila* following knock-down of *dMME1* (Fig. 5B). It is possible that the changes of $Mg^{2+}$ are too subtle under these circumstances to significantly affect the mitochondrial $Ca^{2+}$ homeostasis.

Having shown that reduced expression of *dMME1* led to increased mitochondrial and decreased post-mitochondrial $Mg^{2+}$ levels, we then wondered whether this would result in any physiological consequences to the host organism. While we did not detect any significant aberrance in aspects such as viability, morphology, movement, and fertility, ubiquitous knock-down of *dMME1* caused the flies to live significantly shorter, indicating a general reduction in vitality. As shown

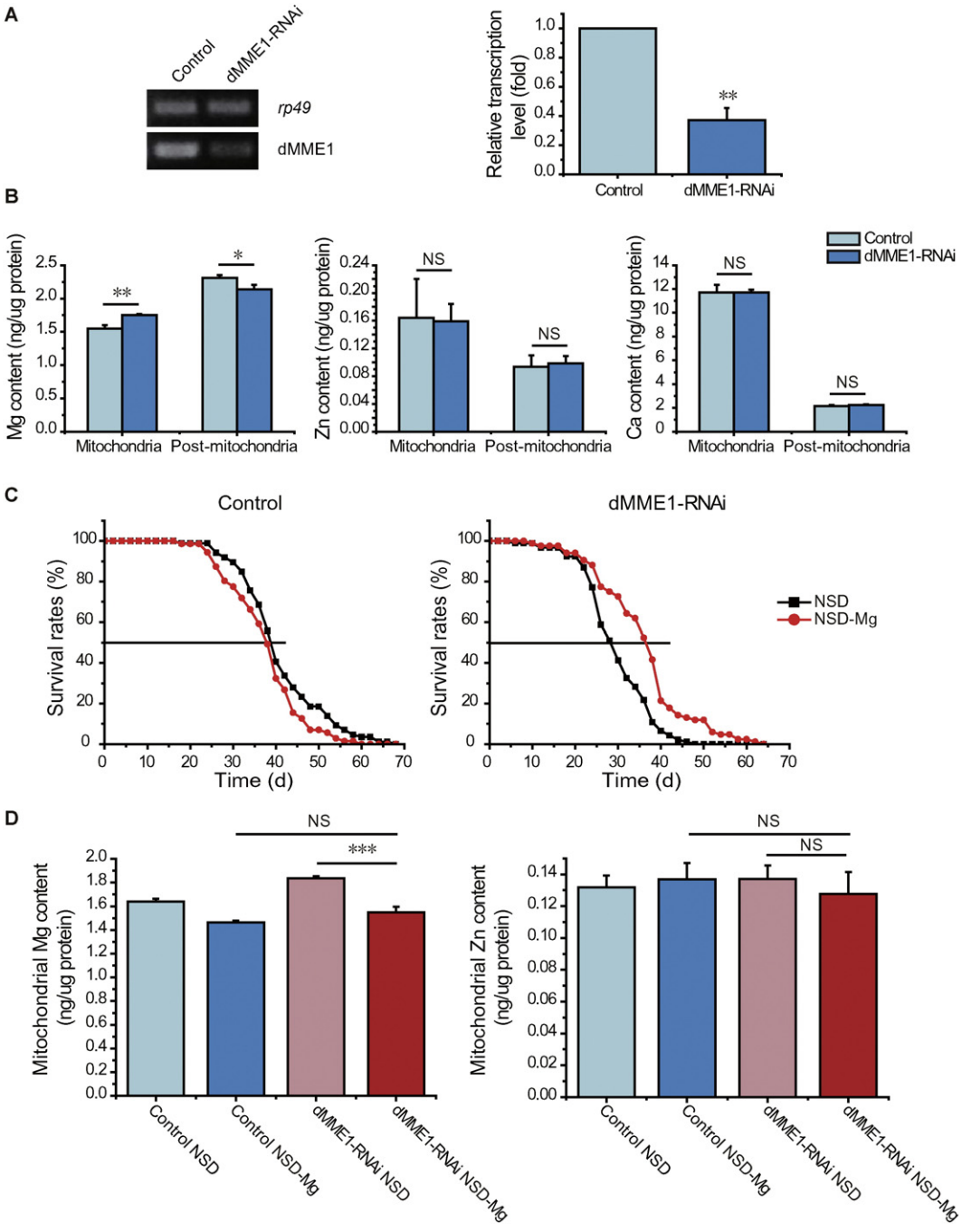


**Fig. 5.** *dMME1* knock-down in *Drosophila* leads to an increase of mitochondrial $Mg^{2+}$. (A) Efficiency of *dMME1* RNAi. Left, RT-PCR analysis of *dMME1* mRNA abundance. *rp49* was used as the loading control. Right, quantification of RT-PCR results from three independent experiments. (B) RNAi of *dMME1* in flies resulted in a significant increase of mitochondrial $Mg^{2+}$ contents and corresponding decrease of post-mitochondrial $Mg^{2+}$ contents. $Zn^{2+}$ and $Ca^{2+}$ contents data are presented side-by-side to show that $Mg^{2+}$ content was relatively specifically affected. Metal contents were determined by ICP-MS. (C) The longevity defect of *dMME1*-RNAi flies could be rescued by $Mg^{2+}$ limitation. Left, life-span of control flies on normal synthetic diet (NSD) or normal synthetic diet with 1/20 $Mg^{2+}$ (NSD-Mg) at 25 °C. The mean life-span of control flies on NSD was 39 days and on NSD-Mg was 38 days. Right, life-span of *dMME1*-RNAi flies on the corresponding foods. The mean life-span of *dMME1*-RNAi flies on NSD was 27 days and on NSD-Mg was 37 days. Log rank test: control flies NSD versus NSD-Mg, NS; *dMME1*-RNAi flies NSD versus NSD-Mg, **; control flies NSD versus *dMME1*-RNAi flies NSD, ***; control flies NSD-Mg versus *dMME1*-RNAi flies NSD-Mg, NS. (D) The increased mitochondrial $Mg^{2+}$ contents in *dMME1*-RNAi flies could be partially depressed by transferring the adult flies from the standard cornmeal media to the synthetic $Mg^{2+}$-deficient food. Metal contents were determined about two weeks after the transferring. The mitochondrial metal contents in B and D are normalized by mitochondrial total protein contents; the post-mitochondrial metal contents in B are normalized by corresponding post-mitochondrial total protein contents. And the data in A, B and D are presented as mean ± S.D.; n ≥ 3. NS stands for no significance.

in Fig. 5C, *dMME1*-RNAi flies exhibited shorter life-spans compared to wild-type control flies on the normal synthetic diet (NSD) [23], whose $Mg^{2+}$ content is controllable. The mean life-span of control flies on the NSD was about 39 days while that of *dMME1*-RNAi flies was only about 27 days (Fig. 5C). In order to assess whether this reduction in longevity was due to increased mitochondrial $Mg^{2+}$ levels, we moved adult flies after eclosion to $Mg^{2+}$-deficient synthetic diet (NSD-Mg). Interestingly and significantly, the survival defect due to ubiquitous *dMME1* knock-down could be fully rescued, supported by an extended mean life-span of the *dMME1*-RNAi flies to about 37 days, which was comparable to the 38 days of the control flies (Fig. 5C). Worth noting is that while $Mg^{2+}$-deficiency rescued *dMME1* knock-down flies, it did

not increase the life-span of control flies, if anything it slightly shortened their life-spans (Fig. 5C). This result indicates that the shortened life-span of *dMME1*-RNAi flies on the normal $Mg^{2+}$ diet is primarily due to an impaired $Mg^{2+}$ homeostasis, presumably as a result of $Mg^{2+}$ accumulation in the mitochondria. To further verify this hypothesis, mitochondrial $Mg^{2+}$ contents of the control and *dMME1*-RNAi flies on either normal or $Mg^{2+}$-deficient diets were measured. As shown in Fig. 5D, transferring the *dMME1*-RNAi adult flies after eclosion to NSD-Mg for only about two weeks sharply reduced their mitochondrial $Mg^{2+}$ content to a level comparable to that of the control flies, while the content of $Zn^{2+}$ was not affected appreciably. This result is consistent with the notion that the shortened life-span of the *dMME1*-RNAi arises from mitochondrial $Mg^{2+}$ increase due to *dMME1* knock-down. It is quite notable here that minor alteration of mitochondrial $Mg^{2+}$

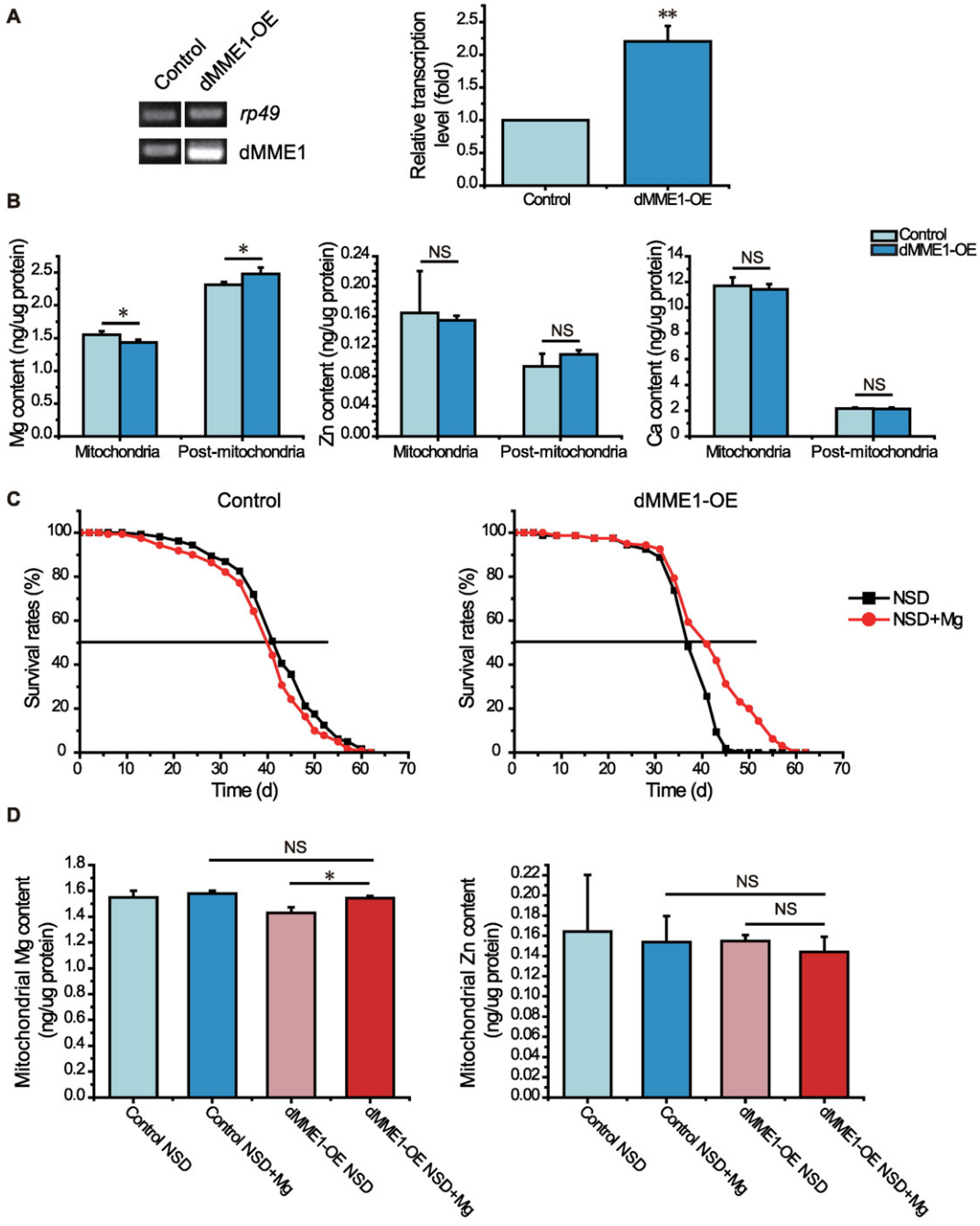


**Fig. 6.** Over-expression of *dMME1* in *Drosophila* leads to a decrease of mitochondrial $Mg^{2+}$. (A) Efficiency of *dMME1* over-expression (*dMME1*-OE). Left, RT-PCR analysis of *dMME1* mRNA abundance. *rp49* was used as the loading control. Right, quantification of RT-PCR results from three independent experiments. (B) Over-expressing *dMME1* resulted in a specific reduction of mitochondrial $Mg^{2+}$ contents and corresponding increase of post-mitochondrial $Mg^{2+}$ contents, but had no much effect on $Zn^{2+}$ or $Ca^{2+}$ contents. (C) The reduced longevity of *dMME1*-OE flies could be rescued by $Mg^{2+}$ supplementation. Left, life-spans of wild-type flies on NSD or NSD adding 2 mM $Mg^{2+}$ (NSD + Mg). The half life-span of control flies on NSD was 41 days and on NSD + Mg was 40 days. Right, life-spans of *dMME1*-OE flies on the corresponding foods. The half life-span of *dMME1*-OE on NSD was 36 days and on NSD + Mg was 41 days. Log rank test: control flies NSD versus NSD + Mg, NS; *dMME1*-OE flies NSD versus NSD + Mg, *; control flies NSD versus *dMME1*-OE flies NSD, **; control flies NSD + Mg versus *dMME1*-OE flies NSD + Mg, NS. (D) The decreased mitochondrial $Mg^{2+}$ contents in *dMME1*-OE flies could be significantly rescued by culturing the files on NSD + Mg. Metal contents were determined about two weeks after transferring the adult fly from the standard cornmeal media to the corresponding synthetic diet. The mitochondrial metal contents in B and D are normalized by mitochondrial total protein contents; the post-mitochondrial metal contents in B are normalized by corresponding post-mitochondrial total protein contents. And the data in A, B and D are presented as mean ± S.D.; n ≥ 3. NS stands for no significance.

could result in obvious physiological consequences, suggesting the necessity of the existence of a cellular mechanism to fine-tune mitochondrial $Mg^{2+}$ homeostasis.

### 2.5. dMme1 over-expression leads to decreased mitochondrial $Mg^{2+}$ contents and a shortened life-span in Drosophila

In order to examine the over-expression effects of *dMME1*, we crossed a fly *dMME1* EP line (an EP line is derived from transposon-generated *Drosophila* strains whose insertion-adjacent gene can be activated by Gal4) to the ubiquitously expressing *Actin-GAL4* flies. RT-PCR analysis revealed that there was a 1.2-fold increase of *dMME1* expression (Fig. 6A). Consistent with the $Mg^{2+}$ exporting function of dMme1, the mitochondrial $Mg^{2+}$ content of the *dMME1*-over-expression (*dMME1*-OE) flies was significantly lower and the post-mitochondrial $Mg^{2+}$ was correspondingly higher than the control flies, opposite to the findings of the *dMME1*-RNAi flies (Figs. 6B and 5B). The levels of both $Zn^{2+}$ and $Ca^{2+}$ were not significantly changed (Fig. 6B). This modest change in $Mg^{2+}$ level was sufficient in shortening *Drosophila* life-span: the mean life-span of the control flies on NSD was about 41 days, while that of the *dMME1*-OE flies was about 36 days (Fig. 6C). To be certain that the shortened longevity of *dMME1*-OE flies was indeed caused by a decrease of the mitochondrial $Mg^{2+}$ content, additional $Mg^{2+}$ was supplemented in NSD diet (NSD + Mg) to test whether it could rescue this defect. Significantly, when the *dMME1*-OE flies were cultivated with NSD + Mg, the survival defect due to ubiquitous over-expression of *dMME1* could be fully rescued: the mean life-span of the *dMME1*-OE flies was extended to 41 days, compared to the life-span of control flies under identical conditions of 40 days (Fig. 6C). This result indicates that the shortened life-span of *dMME1*-OE flies on the normal $Mg^{2+}$ diet may be a result of $Mg^{2+}$ insufficiency in the mitochondria. Consistently, when the mitochondrial $Mg^{2+}$ contents of the control and the *dMME1*-OE flies on either NSD or NSD + Mg were measured, NSD + Mg could specifically restore the mitochondrial $Mg^{2+}$ contents of the *dMME1*-OE flies to the level of the normal flies (Fig. 6D). As a control, the mitochondrial $Zn^{2+}$ levels did not change significantly (Fig. 6D).

Taken together, alteration of *dMME1* expression could perturb the mitochondrial $Mg^{2+}$ homeostasis, leading to modest changes of mitochondrial $Mg^{2+}$ in either direction. These mitochondrial $Mg^{2+}$ changes are accompanied with significant survival reduction of the flies.

### 2.6. Heterologous expression of yeast Mme1 in Drosophila causes an eclosion defect that is rescued by dMme1 knock-down

When the yeast mitochondrial $Mg^{2+}$ exporter Mme1 was heterologously and strongly expressed in the fly, a very extreme phenotype was observed; all the yeast *MME1*-over-expression (*MME1*-OE) flies died very early (at or before 1st larvae). It was possible that yeast Mme1 is a more potent $Mg^{2+}$ exporter than dMme1, or alternatively because yeast Mme1 was more strongly expressed in the fly than dMme1 (dMme1 over-expression was made by the use of an EP line, with only 1.2 fold of expression increase (Fig. 6A)). We thus attempted a local expression of yeast *MME1*. With a muscle-specific driver (*mhc-GAL4*), flies with yeast *MME1* expression could survive the larval stage, and partially eclose to adults (Fig. 7A). The eclosion rate could be significantly increased by additionally knocking-down *dMME1* (Fig. 7A). Meanwhile, the decreased mitochondrial $Mg^{2+}$ contents in flies expressing yeast *MME1* were significantly rescued by *dMME1* knock-down (Fig. 7B). Therefore, in addition to the evidence of a functional complementation between dMme1 and yeast Mme1 in yeast (Fig. 1), we also show that these two gene act analogously in *Drosophila*, further supporting that they are orthologous genes from different organisms.

## 3. Discussion

Here we identified the *Drosophila* orthologue of yeast Mme1, CG3476 (dMme1) and characterized its role in maintaining mitochondrial $Mg^{2+}$ homeostasis. These studies offered us a glimpse into the subtlety of mitochondrial $Mg^{2+}$ homeostasis in a multicellular organism: while presumably regulation of both importing and exporting can be utilized to maintain mitochondrial $Mg^{2+}$ levels, a disruption of the exporting process alone is sufficient to cause modest mitochondrial $Mg^{2+}$ changes, resulting in severe effects on the viability of the organism.

### 3.1. Identification of the Mme1 orthologue in Drosophila

Mitochondrial $Mg^{2+}$ homeostasis is a combined effect of both exporters and importers. We had previously identified a yeast mitochondrial $Mg^{2+}$ exporter, Mme1, by genetic screening [19], and here discovered the *Drosophila* orthologue of yeast Mme1, dMme1, by

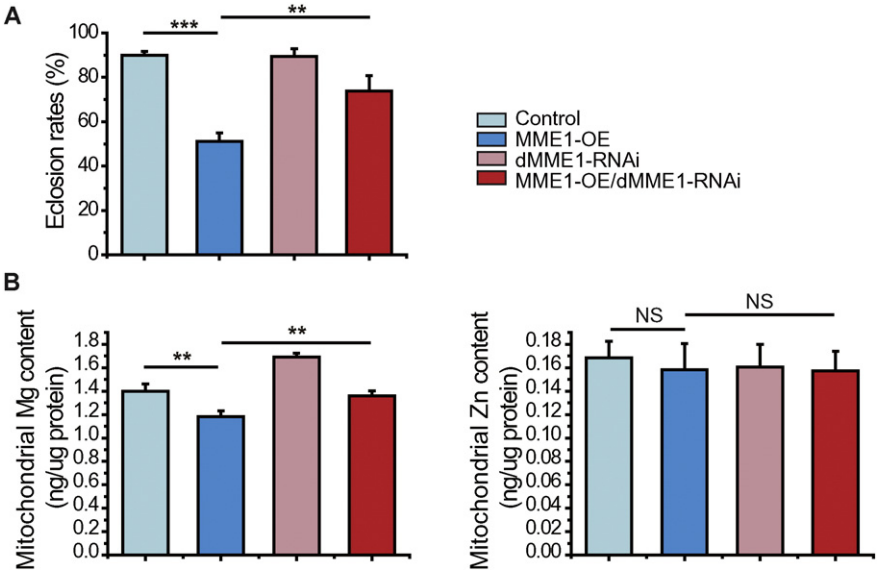


**Fig. 7.** Yeast and fly *MME1*s function analogously in *Drosophila*. (A) The reduced eclosion rate of muscle-expressing yeast *MME1* larvae could be partially rescued by suppression of *dMME1*. Muscle-specific expression was directed by the *mhc-GAL4* driver. Eggs were placed on grape-juice plates and newly hatched progenies were transferred to normal food. Percentages of the fly eclosing to adults (eclosion rate) were calculated. (B) The decreased mitochondrial $Mg^{2+}$ contents in muscle-expressing yeast *MME1* larvae could be significantly rescued by knocking-down of *dMME1*. Mitochondrial $Zn^{2+}$ contents are presented side-by-side as a negative control. Values are presented as mean ± S.D.; n ≥ 3. NS stands for no significance.

homologous comparison and functional complementation. The close resemblance shared by mitochondrial family members prohibited a direct isolation of *Drosophila* Mme1 from a number of highly homologous candidates. In combination with functional complementation in yeast, we were able to identify *dMME1* as the gene involved in mitochondrial $Mg^{2+}$ efflux.

*Drosophila* is an excellent model organism for functional analysis. It has been successfully used in studying the homeostasis of metal ions such as zinc [24–29], iron [30–36], copper [37,38] and even aluminum [39,40], but little is known about its $Mg^{2+}$ homeostasis. Although it is known that in yeast, Mrs2 and Lpe10 form heteropolymers to mediate the influx of mitochondrial $Mg^{2+}$ [18], mitochondrial $Mg^{2+}$ uptakers in *Drosophila* remain to be identified. Besides the mitochondrial $Mg^{2+}$ importers, it is quite likely that other $Mg^{2+}$ transporters, such as those localized to the plasma membrane or intracellular organelle membranes in *Drosophila* or other organisms may be identified in the future by similar methods.

### 3.2. An accurate regulation of $Mg^{2+}$ homeostasis in Drosophila mitochondria

Mitochondrial $Mg^{2+}$ is involved in many processes. It is required for several critical enzymatic reactions in the electron transport chain. Anion channels, such as $H^+$ gradients and membrane potentials across mitochondrial membranes are modulated by mitochondrial $Mg^{2+}$ [1]. $Mg^{2+}$ is also critical for the splicing of group II intron RNA within mitochondria [20].

In yeast, mutations of mitochondrial $Mg^{2+}$ importers, *mrs2Δ* or *lpe10Δ*, or the exporter *MME1* over-expression strain cause a decrease of mitochondrial $Mg^{2+}$ and growth defects on non-fermentable media [13,14,19]. On the other hand, although the deletion of yeast *MME1* leads to an increase of mitochondrial $Mg^{2+}$ contents, no other obvious phenotypes, such as growth defects have been observed [19]. It seems that yeast cells are sensitive to $Mg^{2+}$ shortage but somewhat tolerant of $Mg^{2+}$ overloading. However, this is quite different in *Drosophila*. Perturbations of the expression of the *Drosophila* mitochondrial $Mg^{2+}$ exporter, dMme1, by either over-expression or knock-down, result in only about a 10% change in mitochondrial $Mg^{2+}$ levels in respective directions, but sufficient to lead to obvious physiological consequences such as a shortened life-span. From this, it is apparent that maintaining a tight range of mitochondrial $Mg^{2+}$ concentration is critical to ensure an optimal state for the survival of the whole organism. Some subtle mitochondrial $Mg^{2+}$ dyshomeostasis that may not be significantly harmful to single cellular organisms such as yeast will become detrimental to a multicellular organism such as *Drosophila*. In this aspect, studies in multicellular organisms would offer more complete and accurate reflections of the physiological roles played by $Mg^{2+}$ homeostasis.

## 4. Materials and methods

### 4.1. Plasmids

Expression plasmids used in yeast were pGPD-413 and pTEF-425 obtained from ATCC [41]. The construct used to determine the intracellular localizations of dMme1 in human CHO cells was pEGFP-N1-*dMME1*, constructed in pEGFP-N1 (Clontech, USA) by fusing *dMME1* in frame to the N-terminal of EGFP using the following primers: pEGFP-N1-*dMME1* F: 5′-CGGCTCGAGTCAGCAATGGAGGAGGTCGAAATCAG-3′, pEGFP-N1-*dMME1* R: 5′-CGGGGATCCGGAGCCTTCAGCAAATCATTGG-3′. The construct used in *E. coli* assays was pET15b-*dMME1*, generated by cloning *dMME1* into pET15b (Novagen, Germany) using the following primers: pET15b-*dMME1* F: 5′-CGGCATATGCCGATGGAGGAGGTC GAAATCAG-3′, pET15b-*dMME1* R: 5′-CGGGGATCCTTAAGCCTTCAGCAA ATCAT-3′, and then transformed into *E. coli* BL21 (DE3).

All constructs were verified by sequencing.

### 4.2. Yeast strains and media

Yeast strains used in this study are listed in Table 1. Unless otherwise noted, standard yeast media and growth conditions were applied. Yeast transformation was carried out by the standard lithium acetate method [42]. Empty vectors were also transformed in parallel to serve as negative controls. Single colonies of each transformed yeast strain were picked out and grown on synthetic selective media. Glucose (2%) was added for the SD medium, while 2% glycerol was added for the SG medium.

### 4.3. Fly stocks, culture media, and transgenics

For experiments using adult flies, fly larvae were initially reared on standard cornmeal media at 25 °C to obtain enough fly adults and then transferred to the corresponding synthetic diet for longevity or metal contents determination, as eclosion rates are generally very low on the normal synthetic diets (NSD). NSD were prepared according to Troen AM, et al. [23]. The composition of 1 l synthetic medium was: 1.35 g L-methionine, 150 g glucose, 2.5 g L-aspartic acid, 5.6 g L-glutamic acid, 2.3 g L-serine, 1.0 g Glycine, 1.1 g L-histidine HCl-$H_2O$, 3.9 g L-arginine HCl, 2.1 g L-threonine, 2.6 g L-alanine, 2.1 g L-proline, 1.9 g L-tyrosine, 3.0 g L-valine, 1.0 g L-cystine, 1.9 g L-isoleucine, 3.1 g L-leucine, 2.2 g L-phenylalanine, 6.5 g L-lysine HCl, 17.4 g L-tryptophan, 18.8 μg Vitamin B12, 0.015 μg Biotin, 2 mg P-aminobenzoic acid (PABA), 42 mg Inositol, 10 mg Niacin, 6 mg Pantothenic acid, 6 mg Folic acid, 3 mg Pyridoxine HCl, 2.4 mg Riboflavin, 1.5 mg Thiamin HCl, 16 mg Choline, 1350 IU Vitamin A, 16.5 IU Vitamin E, 335 IU Vitamin D, 0.5 mg Vitamin K, 11.284 mg Zinc, 4.675 mg Copper, 0.5616 mg Chromium, 446.1372 mg Potassium, 245.6724 mg Phosphorus, 4.6569 mg Calcium, 16.0476 mg Chloride, 2.58 mg Iron, 3.185 mg Manganese, 5.0826 mg Sodium, 10 mg Cholesterol, 0.1 g Lecithin and 1.0 g Ribonucleic acid. 24.2556 mg $Mg^{2+}$ was added to generate the NSD, while 1.2128 mg (1/20) $Mg^{2+}$ was added for the deficient food (NSD-Mg), and additional 2 mM $Mg^{2+}$ was supplemented to NSD + Mg food. The purity of all reagents was more than 99.99%. The food was prepared with ultra-pure water and containers clean enough to avoid any additional $Mg^{2+}$ contamination.

The flies used in this study are listed in Table 2. Transgenic flies for the pUAST-*MME1* construct were generated in $w^{1118}$ background by P-element-mediated transformation.

### 4.4. Spotting assays

Yeast strains were adjusted to the identical optical density $(OD)_{600}$ of about 0.5, and then serially diluted to $10^{-1}$, $10^{-2}$, and $10^{-3}$. 3 μl of each diluted yeast culture was spotted on selective SG plates (glycerol as the carbon source). SD plates (glucose as the carbon source) served as the spotting control. Generally, pictures were taken after 2 or 3 days, depending on the growth.

### 4.5. Measurement of metal contents

Isolation of yeast mitochondria was performed according to a previous protocol [43] and isolation of *Drosophila* mitochondria was performed as described [44]. For determination of post-mitochondrial metal contents, post-mitochondria fractions were further spun down by high-speed centrifugation and then the supernatants were used and tested. All the samples were normalized by protein levels, which

**Table 1**
Yeast strains used in this study.

| Strains | Genotype | Source |
|---|---|---|
| BY4742 | MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* | Invitrogen |
| *mme1Δ* | MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 MME1*::kanMX4 | This study |
| *mrs2Δ* | *mrs2*::LEU2 | |

**Table 2**
Fly lines used in this study.

| *Drosophila* | Descriptions | Origin |
|---|---|---|
| *Actin-Gal4/Cyo* (#4414) | Ubiquitous Gal4 | Bloomington *Drosophila* Stock Center |
| *mhc-Gal4* | Muscle-specific Gal4 | Bloomington *Drosophila* Stock Center |
| *dMME1*-OE (#27001) | *CG3476* over-expression line | Bloomington *Drosophila* Stock Center |
| *dMME1*-RNAi (#2734) | *CG3476* RNAi line | Vienna *Drosophila* RNAi Center |
| *MME1*-OE | Yeast *MME1* over-expression line | This study |

were determined by the BCA Protein Assay Kit (Thermo Scientific Pierce, Waltham, MA, USA). The contents of metal ions in each sample were determined by inductively coupled plasma mass spectrometry (ICP-MS) XII (Thermo Electron Corp., Waltham, MA, USA) by the Analysis Center of Tsinghua University. About 0.8 g (dry weight) *E. coli* was used to measure the metal contents in *E. coli*.

### 4.6. RT-PCR

RT-PCR assays to test group II RNA splicing in mitochondria were performed mainly as described previously [15,17]. The RT-PCR assay for *Drosophila* was performed according to Xiaoxi Wang et al. [27]. Briefly, total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was then reverse-transcribed from 1 μg total RNA with Reverse Transcriptase (TransGen Biotech Co., Beijing, China). Semi-quantitative RT-PCR was performed using gene-specific primers to amplify partial regions of target genes. RNA isolation and reverse transcription were performed independently three times, and no less than three RT-PCR experiments were applied to each cDNA sample.

### 4.7. Eclosion and longevity assays

To examine the effects of *dMME1*-RNAi on the eclosion of *MME1*-OE, *mhc-GAL4* homozygous flies were crossed with the *dMME1*-RNAi/*MME1*-OE flies, and the progeny were reared on grape-juice food. The density of each vial was controlled to 60 progeny. The total number of emerging adults of each genotype was counted.

For longevity assays, 2-day-old adult males were collected. 20 flies were placed in each food vial and all vials were kept at 25 °C with 60% humidity under a 12-h light–dark cycle. Food vials were changed every two days, and dead flies were counted at that time. 6 parallel group tests were conducted for each genotype.

### 4.8. Immunofluorescence staining and fluorescence microscopy

CHO cells were cultured in Opti-MEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD, USA) at 37°C. Cells were transfected with pEGFP-N1-*dMME1* together with construct of mitochondrial marker, mCherry-C1-Tom22, or ER marker, pDsRed2-ER vector (Clontech, Cat.# 632,409, USA), or Golgi marker, pDsRed-Monomer-Golgi vector (Clontech, Cat.# 632,480, USA), using the lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA). After 36 h, pictures were taken with a Zeiss LSM780 confocal microscope (Zeiss, Germany).

Immunofluorescence staining of *E. coli* cells expressing dMme1 with N-terminal tagged 6×HIS was carried out as described previously [45,46]. Specifically, the dilution ratio of HIS antibody was 1:200. The secondary antibody goat anti-mouse-FITC was diluted 200 times.

### 4.9. Statistical analysis

The flies' longevity data were analyzed by the Log rank test. And all other data were analyzed by the Student t test. Statistical results are presented as means ± S.D. Asterisks indicate critical levels of significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

## Author contributions

Conceived and designed the experiments: YC SZ and BZ. Performed the experiments: YC SZ and XW. Analyzed the data: YC and SZ. Wrote the paper: YC SZ and BZ.

## Competing interests

The authors declare no conflict of interest.

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