이학석사학위논문

초파리에서
MCU 유전자의 기능 연구

Functional study of mitochondrial calcium uniporter in *Drosophila*

2015년 8월

서울대학교 대학원

생명과학부

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ABSTRACT

Functional study of mitochondrial calcium uniporter in *Drosophila*

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Mitochondrial calcium plays a critical role in diverse cellular processes. Normal mitochondrial calcium uptake enhances energy production and facilitates metabolism. However, in pathological conditions, mitochondrial calcium overload promotes cell death. Therefore, mitochondrial calcium uptake should be under tight regulation for mitochondrial and cellular homeostasis. Recently, mitochondrial calcium uniporter (MCU) was identified as the transporter for rapid mitochondrial calcium uptake. Although there are many assumptions about the physiological roles of MCU, its functions and interactions in vivo are largely unknown. In this study, I generated MCU deletion fly line, which I named MCU^{52}, with loss of MCU gene expression and impaired mitochondrial calcium uptake. I tried to reveal the physiological role of MCU in *Drosophila* by

characterizing the mutant phenotypes. I found that MCU^{52} mutants were more resistant to oxidative stress conditions and defective in locomotion while normal in organism development, mitochondrial dynamics, ATP production, and basal metabolism. Strikingly, in oxidative stress conditions, survival of the MCU^{52} mutants was greater than that of the control. Consistently, MCU^{52} mutants showed reduced cell death after direct H_2O_2 treatment to the larval tissues including the brain and muscles. Collectively, these results demonstrate that MCU contributes to cell death induction upon oxidative stress by sensitizing the cells to mitochondrial calcium overload.

Keywords: MCU, mitochondria, mitochondrial calcium, oxidative stress

Student ID: 2013-22950

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INTRODUCTION

Mitochondria, the organelle for intracellular energy production and cell death signaling, also act as an intracellular calcium reservoir. Endoplasmic reticulum (ER), the largest intracellular calcium reservoir, make close contacts with mitochondria, building the mitochondria-associated ER membrane (MAM) (Fig 1A). Rapid mitochondrial calcium uptake requires such ER-mitochondria junction which provides microdomains of high calcium level upon calcium release from ER. Several proteins at the MAM play critical roles in controlling calcium release from ER to mitochondria. Phosphatidylinositol triphosphate receptor (IP3R) on the ER membrane is responsible for calcium release from the ER side. Calcium ions released from ER are transferred to mitochondria through voltage-dependent anion channel (VDAC) on the outer mitochondrial membrane (OMM) and mitochondrial calcium uniporter (MCU) on the inner mitochondrial membrane (IMM). The molecular chaperone, glucose-regulated protein 75 (grp75) links ER and mitochondria at the MAM through interaction with both IP3R and VDAC (Szabadkai et al., 2006). In addition, mitofusin2 (Mfn2) on the membranes of both ER and mitochondria mediates ER-mitochondria tethering (de Brito and Scorrano, 2008).

The OMM is permeable to various small molecules of 5,000 daltons or less, including calcium ion. Therefore, calcium ions diffuse into the OMM along the electrochemical gradient. Voltage-dependent

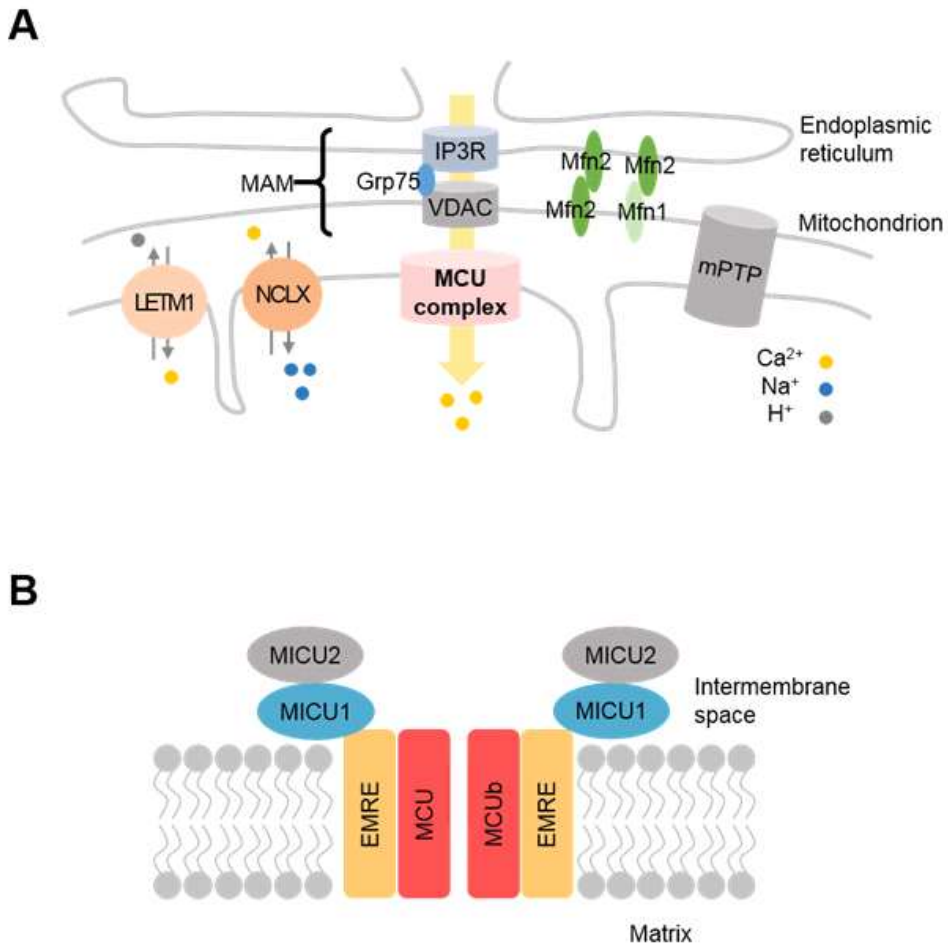


Figure 1. Mitochondrial calcium uniporter complex regulates mitochondrial calcium signaling. (A) Schematic presentation of ER-mitochondria calcium transfer and mitochondrial calcium transporters. (B) The components of mitochondrial calcium uniporter complex in mammals.

anion channel 1 (VDAC1), a beta barrel channel protein, forms a large pore in the OMM, allowing efficient influx of calcium ions. The IMM is impermeable to ions, and various types of transporters located in the IMM contribute to mitochondrial calcium dynamics across the membrane. Mitochondria maintain basal calcium ion concentration of ~0.1 μM due to the mitochondrial membrane potential. When calcium is release from ER in response to stimuli, mitochondria rapidly uptake calcium ions through MCU, a highly selective and Ruthenium Red-sensitive transporter. Low affinity of MCU can be overcome with high calcium concentration at the MAM when ER releases calcium.

The phenomenon that energized mitochondria rapidly uptake calcium ions has been reported over 50 years, however the molecular identity of mitochondrial calcium uniporter was revealed only a few years ago. MICU1 was the first to be identified as a component of mitochondrial uniporter complex (Perocchi et al., 2010). A year later, the gene encoding the pore of mitochondrial calcium uniporter, MCU, was discovered by two groups using in silico methods (Baughman et al., 2011; De Stefani et al., 2011). Soon after, MICU2 (Plovanich et al., 2013), MCUb (Raffaello et al., 2013), EMRE (Sancak et al., 2013), and SLC25A23 (Hoffman et al., 2014) were suggested as other subunits which consist a complex with MCU and MICU1 (Fig 1B).

Mitochondrial calcium participates in a broad range of intracellular processes from energy production to cell death. Sufficient mitochondrial calcium level contributes to enhanced ATP synthesis,

stimulating three dehydrogenases acting in the TCA cycle. However, mitochondrial calcium overload in pathological conditions results in increased reactive oxygen species (ROS) production and triggers opening of the mitochondrial permeability transition pore (mPTP). Transient or sustained opening of the mPTP results in apoptotic or necrotic cell death. In addition, mitochondria also act as a buffer in cytoplasmic calcium signaling by rapidly taking up local calcium from the cytosol. Therefore, mitochondrial calcium uptake through MCU should be under tight regulation to maintain both mitochondrial and cellular calcium homeostasis.

Identification of the components of the MCU complex accelerated the research about MCU and mitochondrial calcium uptake. Over the last few years, studies in MCU proposed a diverse role of MCU at cellular and organism levels. Cellular studies of MICU1 showed that MICU1 deficiency resulted in impaired cell migration and increased stimulus-induced cell death (Mallilankaraman et al., 2012). In another study, mitochondrial calcium uptake through MCU was shown to increase excitotoxic cell death in neurons (Qiu et al., 2013). In pancreatic beta cells, MCU was proposed to contribute to metabolism-secretion coupling (Alam et al., 2012; Groschner et al., 2014; Tarasov et al., 2012). Recently, several in vivo studies of MCU and MICU1 gave us insight to the physiological role of mitochondrial calcium uptake. MCU loss-of-function mice exhibited impaired skeletal muscle performance and loss of mPTP-mediated cell death, however there was no significant changes in basal metabolism and

overall cell death (Pan et al., 2013). From the study of MICU1 loss-of-function human patients, MICU1 is involved in mitochondrial dynamics and energy homeostasis and is critical for proper function of nervous system and muscle tissues (Logan et al., 2014). Other researches on MCU and mitochondrial calcium also suggested their unpredicted roles on heart rate acceleration during fight or flight response (Wu et al., 2015), ROS-mediated wound repair (Xu and Chisholm, 2014), skeletal muscle trophism (Pardo et al., 2015), cardiac contractility (Wahlquist et al., 2014; Xu and Chisholm, 2014), and even on the cellular movement during zebrafish gastrulation (Prudent et al., 2013).

It is reported that cytosolic calcium is increased under oxidative stress conditions (Doan et al., 1994; Renard et al., 1992; Roveri et al., 1992), which could trigger mitochondrial calcium increase in turn. Importantly, oxidative stress plays an important role in a variety of disease pathogenesis including neurodegeneration (Lin and Beal, 2006), cardiac diseases (Dhalla et al., 2000), diabetes (Maritim et al., 2003), and muscle dystrophy (Piccolo et al., 1991; Rando, 2002). Defective calcium handling has been reported in many of these diseases- neurodegenerative diseases (Cali et al., 2012; Celsi et al., 2009), cardiac diseases (Luo and Anderson, 2013), diabetes (Patti and Corvera, 2010), muscle dystrophy (Bodensteiner and Engel, 1978), (Whitehead et al., 2006). However, it is unknown whether regulation of mitochondrial calcium and MCU could alleviate oxidative stress responses including mPTP opening and cell death in vivo.

While loss of function studies of MCU in vivo were performed in mouse and C. elegans, there is no previous research of MCU or MCU complex in the *Drosophila* system which provides a powerful tool for genetic studies. In this study, we generated the first MCU loss-of-function model in *Drosophila* and characterized its phenotypes on calcium handling, metabolism, locomotion, cell death, and oxidative stress, demonstrating that MCU is critical for oxidative response in *Drosophila*.

MATERIALS and METHODS

Fly strains

MCU^{52} mutant was generated using imprecise P element excision of GS11565 line obtained from DGRC Kyoto. Revertant was generated from precise P element excision of the same strain. Excisions were confirmed by PCR using primers of following sequences : 5'-GACGGAATTGCGATGGAAAATC-3', 5'-GCCAAAAATCCCAT TCTAGTG-3'. UAS-Ratiometric-Pericam.mt was kindly gifted by Gregory T. Macleod. UAS-GCaMP3.T was obtained from Bloomington Drosophila Stock Center. MCU cDNA LD26402 clone was purchased from Drosophila Genomics Resource Center, Indiana University. UAS-MCU-flag, UAS-hMCU-flag, UAS-$MCU^{\Delta C}$-flag, and UAS-MCU^{NIMQ}-flag were generated by microinjection of pUAST vector-cloned DNA into w^{1118} embryos.

Qunatitative RT-PCR

Total RNA was extracted from wandering larvae using Trizol Reagent (Invitrogen). Extracted RNAs were reverse transcribed and amplified by PCR with primers of the following sequences : 5'-GTCTCGCCCTGCGTTTGG-3', 5'-CGAAGCTTCTGTGCTGCTG-3' for MCU(CG18769) gene and 5'-GCGCTTCTTGGAGGAGACGCCG-3', 5'-GCTTCAACATGACCATCCGCCC-3' for RP49 gene. mRNA levels were normalized by RP49 mRNA levels.

Immunoblot and immunostaining

For immunoblot samples, tissues were homogenized in ice cold lysis buffer (20mM Tris-Cl pH 7.5, 100 mM NaCl, 1 mM EDTA, 2 mM EGTA, 1 mM Na_2VO_4, 50 mM beta-glycerolphosphate, 50 mM NaF, 1% Triton X-100) with pestle. Homogenized samples were incubated in ice for 15min, centrifuged, and denatured. Rabbit anti-flag (Cell Signaling Technology), mouse anti-Drosophila MCU (AbClon), and mouse anti-beta tubulin (DSHB) were used in immunoblot and immunostaining.

Fecundity test

Newly eclosed adult flies were collected and 2 or 3 virgins were mated with 3 males for 48 hours. Eggs were collected for the next 48 hours and the progeny were counted. Each test was repeated at least 3 times.

Muscle sections

Thorax muscle section was performed as previously described (Park et al., 2006).

TUNEL assay

To detect cell death in thorax muscle, thoraces of 3-day-old flies were fixed in 4% paraformaldehyde, washed with PBST, and dissected in longitudinal direction. To detect H_2O_2-induced cell death,

wandering larvae were dissected in Schneider's medium and incubated in Schneider's medium containing 0.1% H_2O_2 for 12 hours. Dissected larvae were fixed in 4% paraformaldehyde and washed with PBS. Samples were incubated in 0.1M sodium citrate at 65℃ and cell death was detected by using In situ cell death detection kit TMR red (Roche).

Climbing assay

Ten 3~5-day-old flies were moved to the bottom of a vial by tapping the vial 3~5 times and time required for five flies to climb 15cm was measured. Files were anesthetized at least 24 hours before the tests and all tests were repeated at least 3 times.

ATP assay and triglyceride assay

To measure ATP levels of thoracic mitochondria, 2 thoraces were dissected from 3-day-old male flies and homogenized in 100㎕ of ice cold lysis buffer. ATP levels were measured using ATP bioluminescence kit HS II (Roche). To measure triglyceride levels of adult flies, three 3~5-day-old female flies were homogenized in 100㎕ of 0.5% Tween20 PBS and incubated at 70℃ for 5min and then transferred to ice. 20㎕ of the homogenized samples was mixed with either the same volume of PBS or triglyceride reagent (Sigma-Aldrich). Samples were incubated at 37℃ for 30min for the hydrolysis of triglycerides to glycerol. Then, the amount of glycerol was measured by using free glycerol reagent (Sigma-Aldrich). The

amount of triglycerides was calculated by subtracting the amount of endogenous free glycerol from the amount of glycerol after the hydrolysis reaction. All assays were repeated at least 3 times for each sample.

Trehalose and glucose assay

Trehalose and glucose measurements were performed as previously described (Tennessen et al., 2014). For each genotype, hemolymph from 5~7 wandering larvae was extracted by tearing the cuticles. 1㎕ of hemolymph was diluted with 99㎕ of trehalase buffer (5mM Tris pH 6.6, 137mM NaCl, 2.7mM KCl) and incubated at 70℃ for 5min. Then, 40㎕ of diluted hemolymph was mixed with either 40㎕ of trehalase buffer or 40㎕ of trehalase solution. Samples were incubated at 37℃ overnight, and glucose levels were measured using Glucose (HK) Assay Kit (Sigma-Aldrich).

Starvation assay

3~5-day-old flies were transferred to vials containing 3M paper soaked with DDW. Flies were kept in density of less than 20 flies per vial, and survived flies were counted and transferred to new vials every 12 hours or shorter intervals.

Starvation-induced autophagy

Third instar larvae before in wandering stage were rinsed with PBS

and either starved in DDW-containing petri dish or fed in food-containing vial for 4 hours. Then, larvae were dissected, fixed in 4% paraformaldehyde, and stained with Hoechst. mCherryATG8 signals were observed under confocal microscope.

H_2O_2 and paraquat survival

3~5-day-old males were starved 4~6 hours in DDW-containing vials and transferred to vials containing either 1% H_2O_2, 5% sucrose or 20mM paraquat, 10% sucrose. Survived flies were transferred to new vials and counted every 24 or 8 hours.

Lifespan measurement

Flies were transferred to new vials and survived flies were counted every 2~3 days. Flies were kept under 70% humidity, 25℃ with density lower than 20 flies per vial.

RESULTS and DISCUSSION

Generation of MCU deletion mutant *Drosophila*

To find the orthologous genes of human MCU complex in *Drosophila*, we performed BLAST search and discovered that 4 genes are conserved in the *Drosophila* genome. (Table 1). For loss-of-function study of MCU in *Drosophila*, we generated MCU deletion mutant which we named MCU^{52} by using imprecise excision of P element. We also generated the revertant (RV) with precise excision for the control. Deletion region of MCU^{52} mutant genome includes the start codon and the first exon of *Drosophila* MCU gene (Fig 2A).

MCU^{52} mutants were viable and developed normally as the revertants (Fig 2B). To confirm the deletion of MCU gene in MCU^{52} mutants, we performed genomic DNA PCR, quantitative RT-PCR of MCU gene, and immunoblot for endogenous MCU. We found genomic deletion within the MCU gene of MCU^{52} mutants (Fig 2C). MCU mRNA and protein were also not detected in these mutant flies (Fig 2D, E). While keeping these mutant files, we found that homozygote MCU^{52} mutants failed to breed. We tested fecundity of MCU^{52} mutants and found that MCU^{52} females had reduced number of progeny and MCU^{52} males were semi-sterile (Fig 2F).

Table 1. *Drosophila* orthologs of the MCU complex

	Human	*Drosophila*
Pore-forming	MCU	CG18769
	MCUb	-
Regulator	MICU1	CG4495
	MICU2,3	CG4662
Bridge	EMRE	CG17680

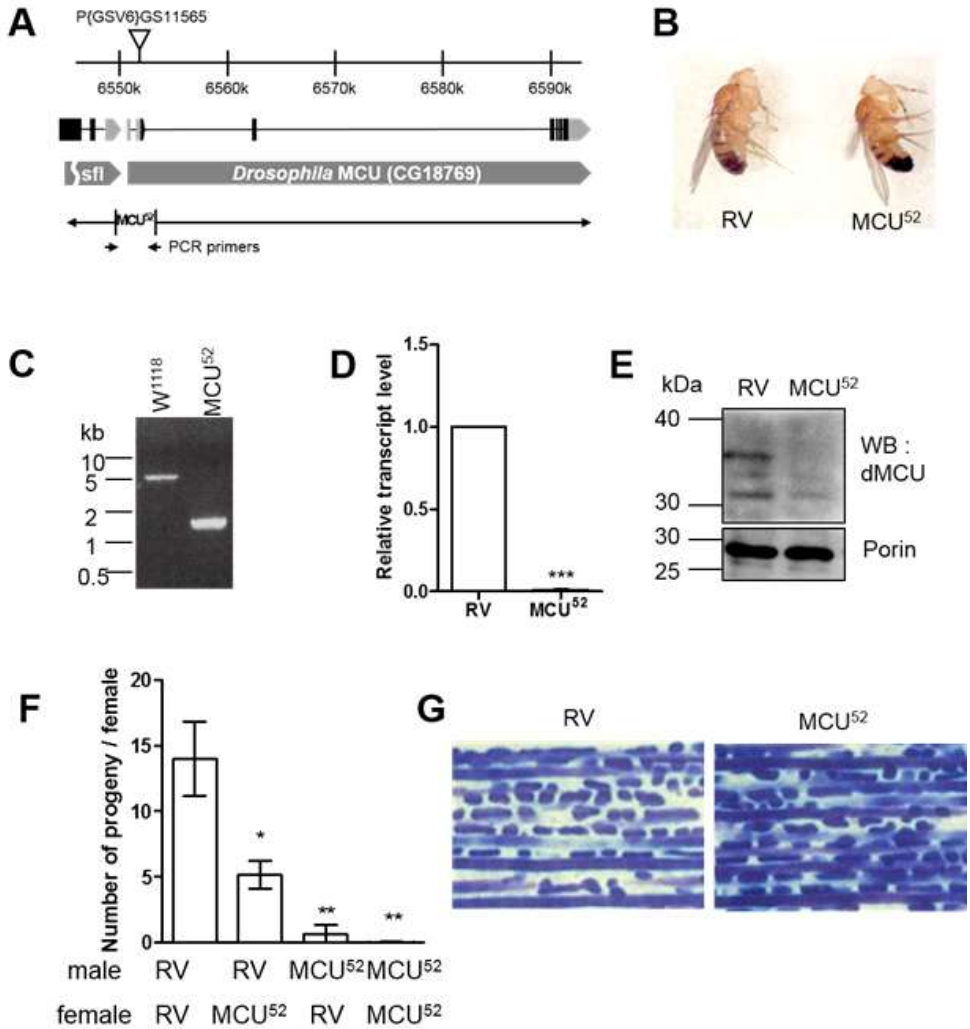


Figure 2. Generation of MCU deletion mutant in *Drosophila*. (A) Genomic map of MCU^{52} mutant. (B) MCU^{52} mutants were viable. (C) Genomic DNA PCR confirmed deletion within the MCU gene in MCU^{52} mutants. (D) Relative mRNA levels of MCU in MCU^{52} mutants and the revertants. (E) Immunoblot of *Drosophila* MCU. (F) Reduced fecundity of MCU^{52} mutants. Mean ± SEM; *p < 0.05, **p < 0.01, n=3~4. (G) Mitochondrial morphology of MCU^{52} mutants.

Impaired mitochondrial calcium uptake in MCU^{52}

To investigate whether mitochondrial calcium uptake is ablated in MCU^{52} mutants, we measured mitochondrial calcium level in muscle tissues upon caffeine treatment. Caffeine is known as an inducer of rapid mitochondrial calcium uptake by stimulating calcium release from ER. A mitochondrial calcium indicator, mito-ratiometric pericam, was expressed in muscle tissues driven by mef2-gal4. In control larvae, mitochondrial calcium concentration was increased upon caffeine stimulation. However, MCU^{52} larvae did not show any increase in mitochondrial calcium upon caffeine stimulation, which was rescued by overexpression of *Drosophila* MCU (Fig 3A-C). We also measured cytoplasmic calcium change upon caffeine stimulation. Cytoplasmic calcium was increased by caffeine and it was quickly recovered to the basal level in both control and MCU^{52} mutant larvae (Fig 3D-F). These results showed that *Drosophila* MCU is required for rapid mitochondrial calcium uptake and MCU^{52} mutant is appropriate to study the in vivo functions of MCU.

Drosophila MCU was functionally equivalent to mammalian MCU

Next, we sought whether *Drosophila* MCU encoded by CG18769 shares biochemical properties of mammalian MCU and is functionally equivalent to mammalian MCU. Like human MCU, *Drosophila* MCU protein has a mitochondrial targeting sequence at its

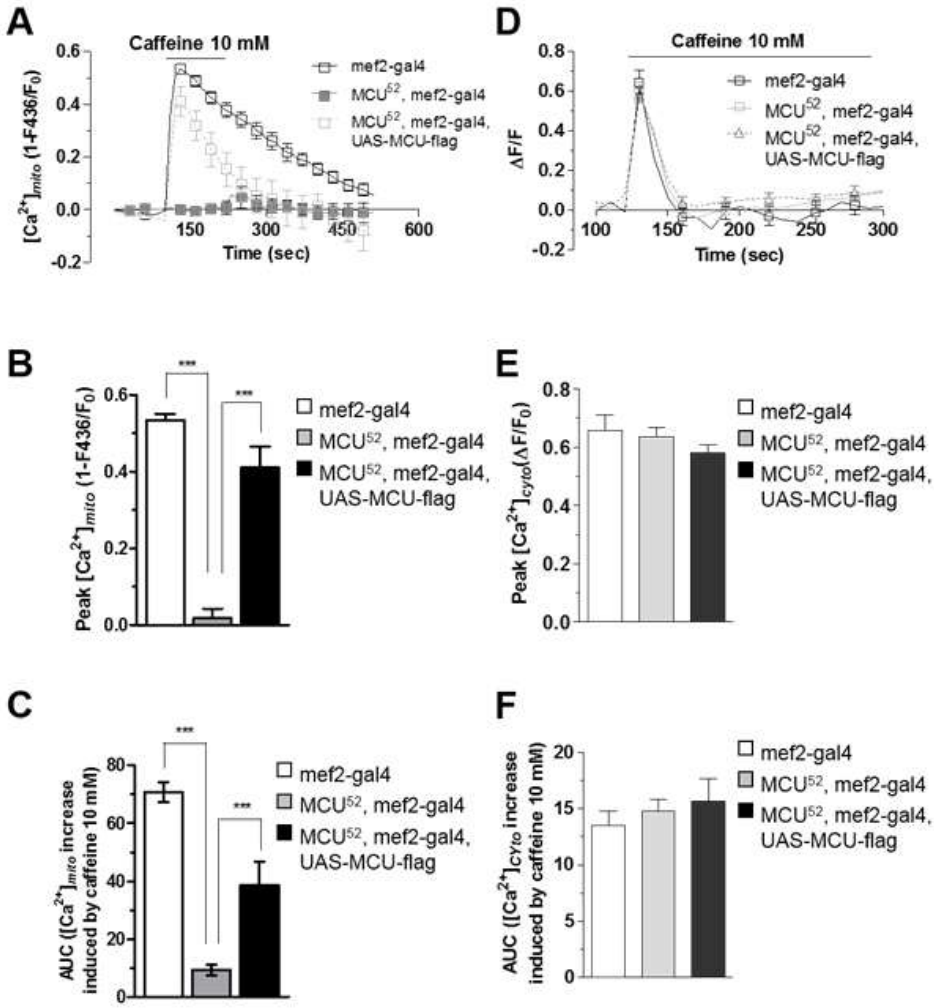

A
Caffeine 10 mM
mef2-gal4
MCU52, mef2-gal4
MCU52, mef2-gal4, UAS-MCU-flag
[Ca2+]mito (1-F436/F0)
Time (sec)
D
Caffeine 10 mM
mef2-gal4
MCU52, mef2-gal4
MCU52, mef2-gal4, UAS-MCU-flag
ΔF/F
Time (sec)
B
Peak [Ca2+]mito (1-F436/F0)
mef2-gal4
MCU52, mef2-gal4
MCU52, mef2-gal4, UAS-MCU-flag
E
Peak [Ca2+]cyto(ΔF/F0)
mef2-gal4
MCU52, mef2-gal4
MCU52, mef2-gal4, UAS-MCU-flag
C
AUC ([Ca2+]mito increase induced by caffeine 10 mM)
mef2-gal4
MCU52, mef2-gal4
MCU52, mef2-gal4, UAS-MCU-flag
F
AUC ([Ca2+]cyto increase induced by caffeine 10 mM)
mef2-gal4
MCU52, mef2-gal4
MCU52, mef2-gal4, UAS-MCU-flag

Figure 3. Impaired mitochondrial calcium uptake in MCU^{52} mutants. (A) Mito-ratiometric pericam signal showed that caffeine-induced mitochondrial calcium uptake was ablated in MCU^{52} mutants and recovered by MCU expression. (B) The peak concentrations of mitochondrial calcium uptake of (A). (C) The area under the curve of (A). (D) GCaMP3 signal showed cytoplasmic calcium increase upon caffeine stimulation. (E) The peak concentrations of cytoplasmic calcium of (D). (F) The area under the curve of (D). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

C terminus, two coiled-coil domains, two transmembrane domains, and the sequence EYSWDIMEPV which is known as the DIME motif (Fig 4).

First, we found *Drosophila* MCU colocalized with mitochondria from immunostaining of larval salivary gland tissues. Overexpressed MCU-flag driven by sgs-gal4 was colocalized with the mitochondrial protein, ATP5A (Fig 5A). Then, we asked if artificial expression of human MCU could recover impaired mitochondrial calcium uptake of MCU^{52} mutants. Overexpression of human MCU in MCU^{52} mutant larvae resulted in increased mitochondrial calcium uptake in muscle tissues upon caffeine stimulation (Fig 5B-D). This indicated that *Drosophila* MCU is a functional ortholog of human MCU.

In previous studies of human MCU, substitution of two acidic amino acids within the DIME motif resulted in a dominant-negative effect on the uniporter activity of MCU (Patron et al., 2014; Raffaello et al., 2013). Another study of human MCU has shown that the MCU mutant with deletion in the coiled-coil domain failed to interact with its regulator MICU1 (Hoffman et al., 2013). I generated transgenic flies of these two mutant forms of MCU - $MCU^{\Delta C}$ and MCU^{NIMQ} (Fig 4), and tested if these mutant forms are functional calcium uniporters. In contrast to wild type, $MCU^{\Delta C}$ or MCU^{NIMQ} expression failed to restore impaired mitochondrial calcium uptake of MCU^{52} (Fig 6). These results demonstrated that the C-terminal coiled-coil and the DIME motif of MCU are essential for its calcium uptake activity.

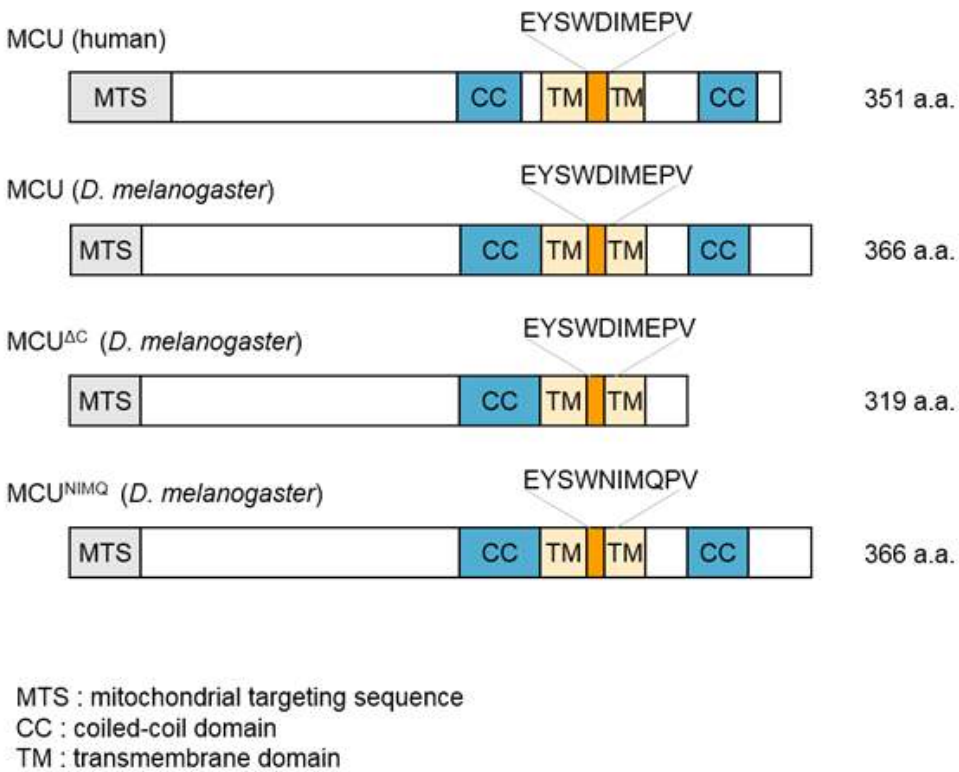


Figure 4. Molecular structures of MCU and its mutant constructs.

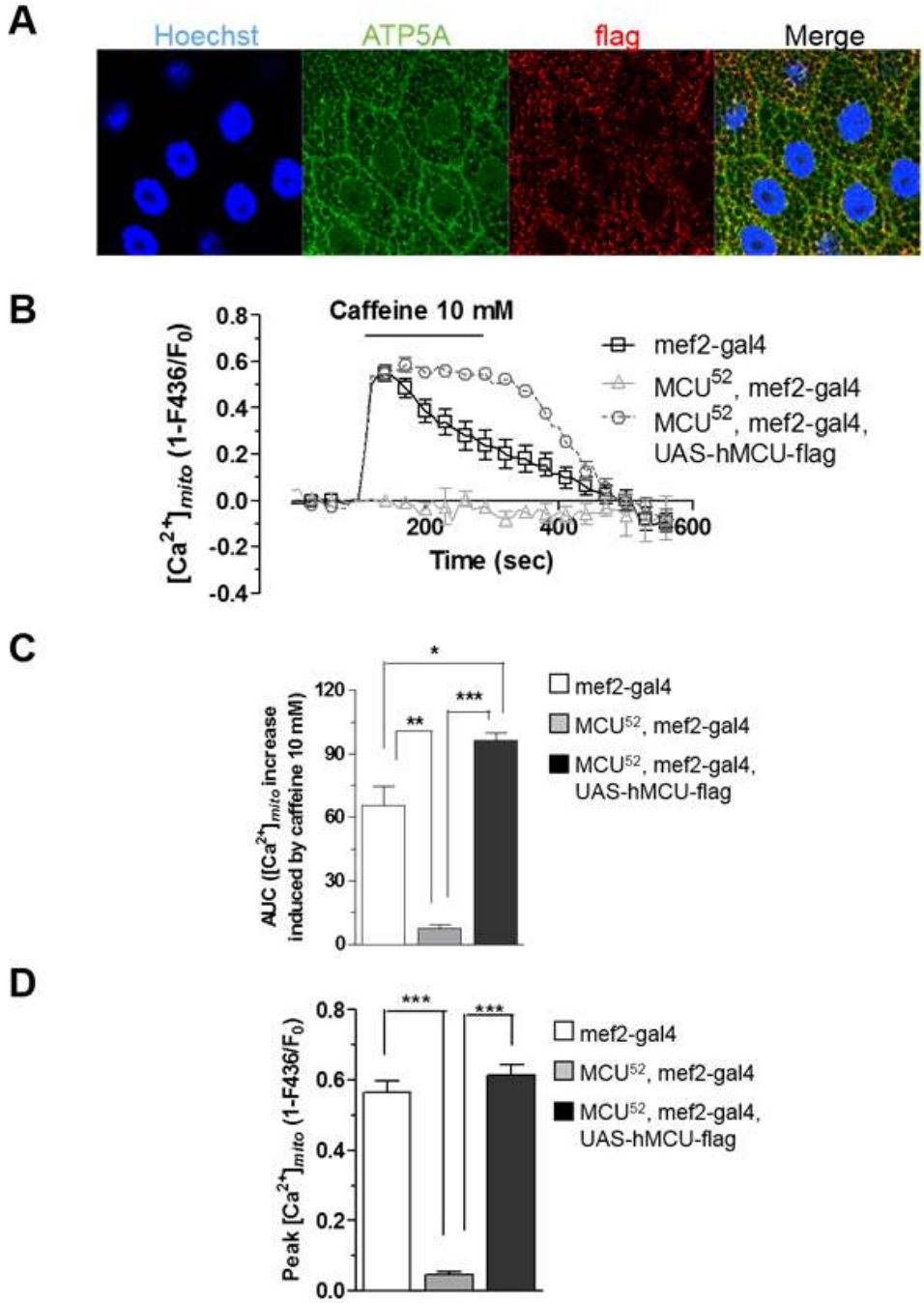

A
Hoechst
ATP5A
flag
Merge
B
Caffeine 10 mM
mef2-gal4
MCU52, mef2-gal4
MCU52, mef2-gal4, UAS-hMCU-flag
[Ca2+]mito (1-F436/F0)
Time (sec)
C
AUC ([Ca2+]mito increase induced by caffeine 10 mM)
D
Peak [Ca2+]mito (1-F436/F0)

Figure 5. *Drosophila* MCU was functionally equivalent to human MCU. (A) *Drosophila* MCU is localized to mitochondria. (B) Impaired mitochondrial calcium uptake of MCU^{52} mutants is recovered with human MCU gene expression. (C) The peak concentration of mitochondrial calcium uptake of (B). (D) The area under the curve of (B). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

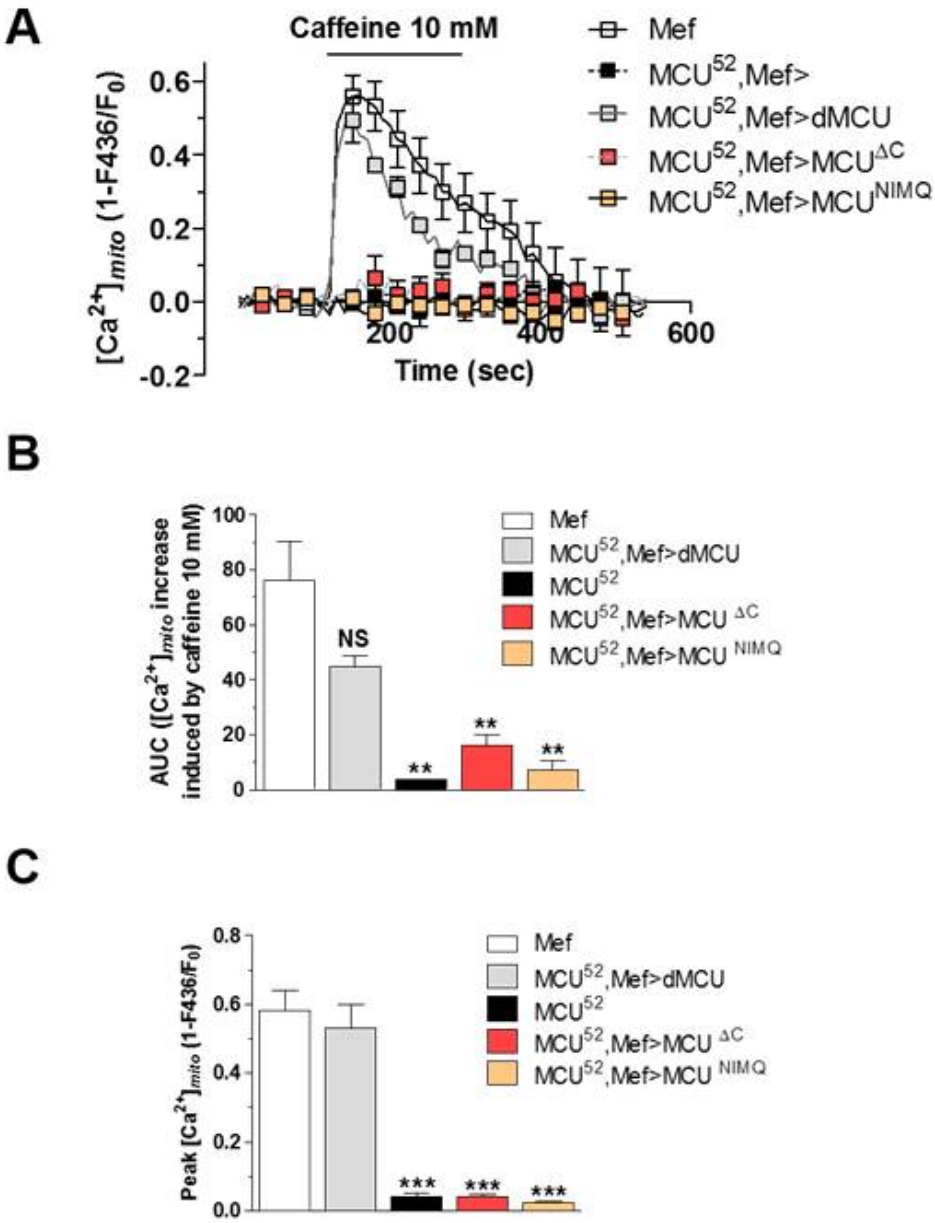

A
Caffeine 10 mM
Mef
MCU52,Mef>
MCU52,Mef>dMCU
MCU52,Mef>MCUΔC
MCU52,Mef>MCUNIMQ
[Ca2+]mito (1-F436/F0)
0.6
0.4
0.2
0.0
-0.2
200
400
600
Time (sec)
B
AUC ([Ca2+]mito increase induced by caffeine 10 mM)
100
80
60
40
20
0
NS
**
**
**
Mef
MCU52,Mef>dMCU
MCU52
MCU52,Mef>MCU ΔC
MCU52,Mef>MCU NIMQ
C
Peak [Ca2+]mito (1-F436/F0)
0.8
0.6
0.4
0.2
0.0
***
***
***
Mef
MCU52,Mef>dMCU
MCU52
MCU52,Mef>MCU ΔC
MCU52,Mef>MCU NIMQ

Figure 6. The C-terminal coiled-coil domain and the DIME motif were essential for MCU activity. (A) Mitochondrial calcium uptake of wild type, MCU^{52}, and MCU^{52} expressing MCU, $MCU^{\Delta C}$ or MCU^{NIMQ}. (B) The area under the curve of (A). (C) The peak concentration of mitochondrial calcium uptake of (A). *p < 0.05, **p < 0.01, ***p <0.001.

MCU overexpression induced mitochondrial fragmentation

To understand the role of mitochondrial calcium uptake in mitochondrial dynamics, we observed mitochondrial morphology in thoracic muscle of MCU^{52} mutants and MCU-overexpressing flies. Thoracic muscle section revealed that MCU^{52} mutants have mitochondria with normal morphology, containing both fragmented and elongated mitochondria as the revertants. However, overexpression of MCU in muscle tissues driven by mef2-gal4 resulted in severe fragmentation of mitochondria (Fig 7).

MCU^{52} mutants were defective in locomotion

Calcium is involved in the process of muscle contraction and, therefore, calcium signaling in muscle tissues is especially critical for the muscle performance. We hypothesized that loss of MCU would alter muscle ability. To test this hypothesis, we measured the locomotor activity of MCU^{52} mutant flies with startle-induced climbing assay. Notably, MCU^{52} mutants showed a mild decrease in climbing speed, which was partially recovered by expression of exogenous *Drosophila* MCU (Fig 8A). We also tested whether overexpression of MCU could enhance the climbing ability. However, overexpression of MCU did not affect the climbing ability of wild type control (Fig 8B).

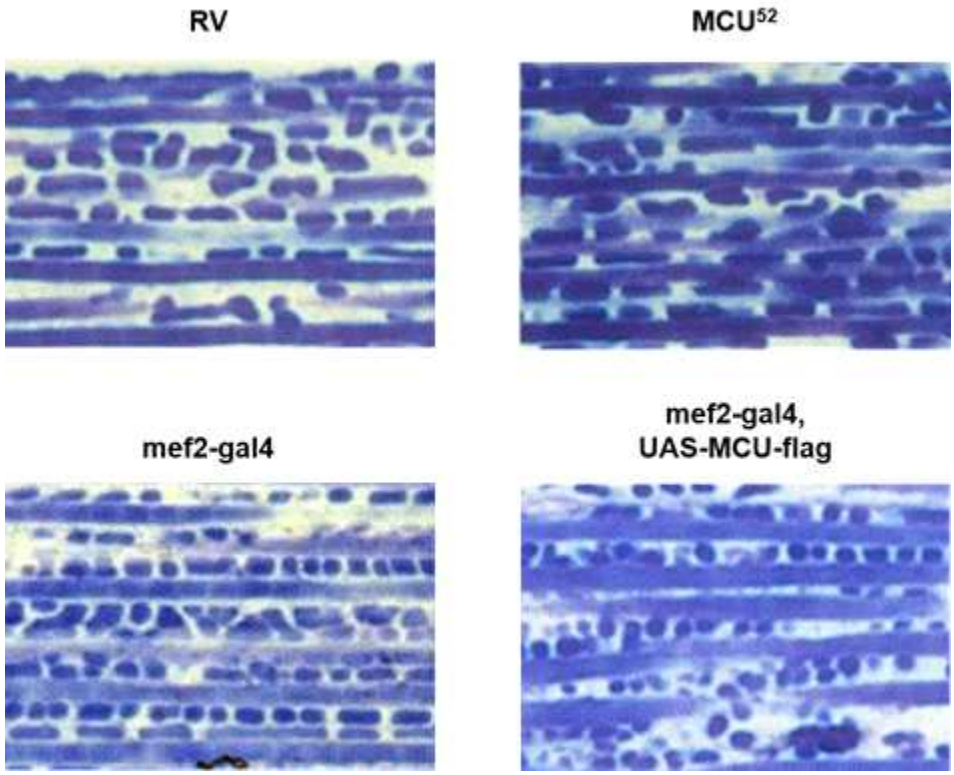


Figure 7. Mitochondrial morphology of MCU^{52} mutants and MCU-overexpressing flies. MCU^{52} mutants showed normal mitochondrial morphology, while overexpression of MCU resulted in mitochondrial fragmentation.

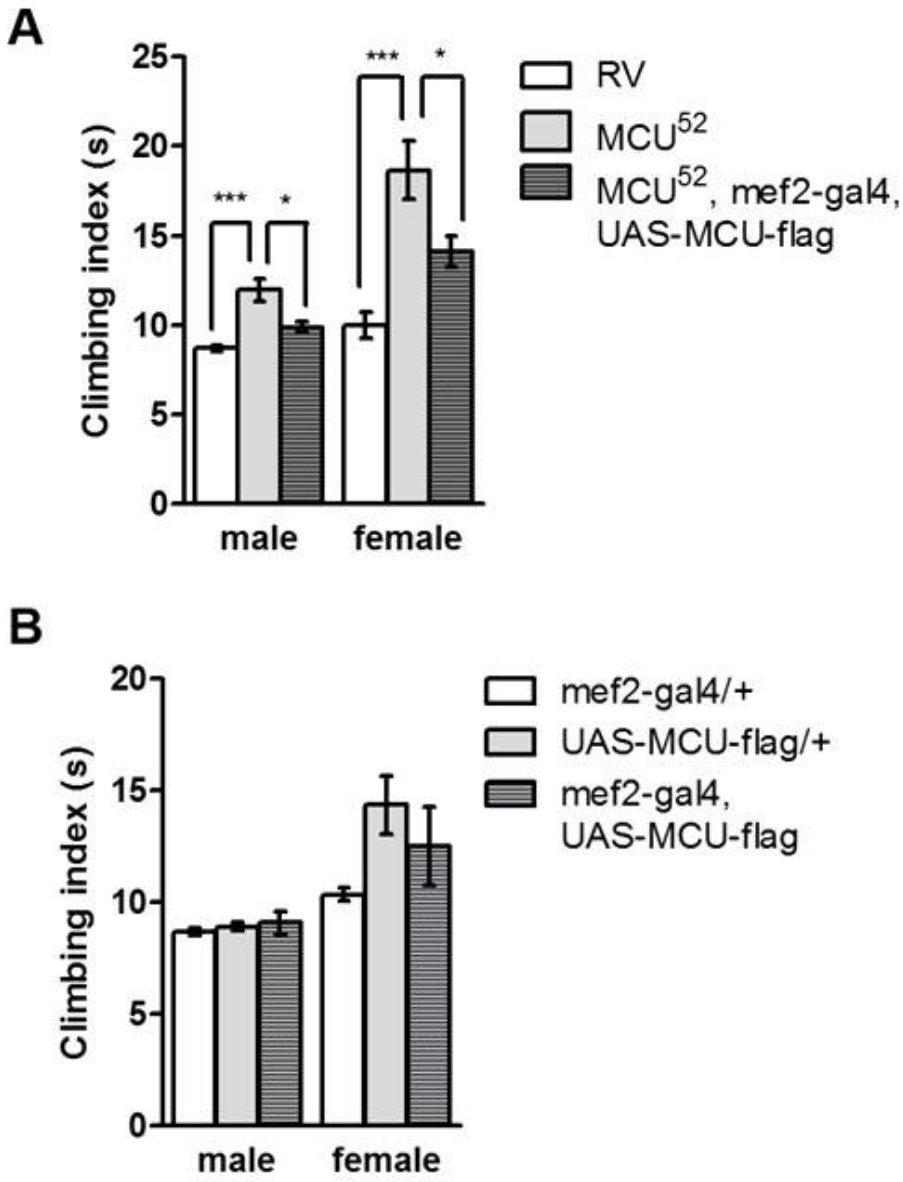


Figure 8. MCU^{52} mutants exhibited reduced startle-induced locomotion. (A) Loss of MCU resulted in impaired locomotor activity. (B) Overexpression of MCU did not enhance climbing ability. Mean ± SEM; $*p < 0.05$, $***p < 0.001$, n=5~7.

Overexpression or loss of MCU did not induce cell death autonomously in muscle tissues

Because mitochondrial calcium overload is known as a signal triggering cell death, we observed cell death of MCU^{52} mutants or MCU-overexpressing flies by using TUNEL assay in thorax muscle tissues. While PINK1 B9 as a positive control exhibited cell death signals in thorax muscle, MCU overexpressing muscle tissues did not show any detectable cell death signal (Fig 9A). MCU^{52} mutants also did not show any cell death in thorax muscles (Fig 9B), indicating its reduced climbing ability is not directly due to muscle cell death.

Metabolic phenotypes of MCU^{52} mutants

Regarding the role of mitochondrial calcium in cell metabolism, we investigated overall metabolic phenotypes of MCU^{52} mutants. Body weight, ATP level, triglyceride level, concentration of circulating sugars in the hemolymph of MCU^{52} mutants were not significantly different from those of the revertants (Fig 10A-D). We also did not observe any detectable changes in the amount of food intake and starvation-induced autophagy (Fig 10E, F, respectively). Upon starvation, survival of female MCU^{52} mutants was indistinguishable from the revertants (Fig 10G), while MCU52 male flies showed increased survival (Fig 10H). Collectively, these results showed that loss of MCU does not alter basal metabolism in *Drosophila*.

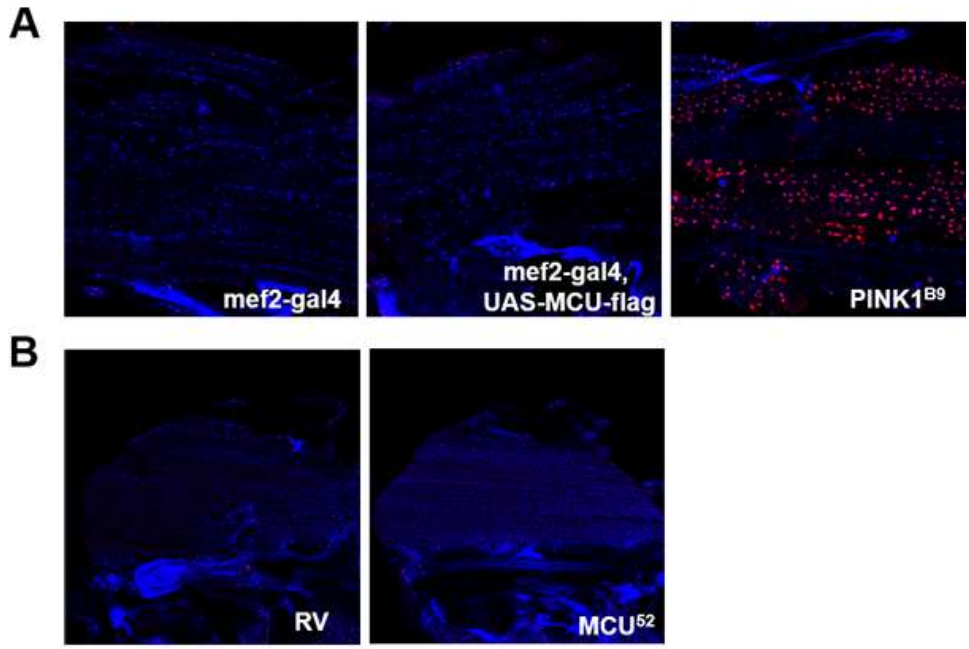


Figure 9. Overexpression or loss of MCU did not induce cell death autonomously in thorax muscle. (A) MCU overexpression in muscle did not induce cell death. TUNEL (red), Hoechst (blue). (B) No detectable cell death occurred in the muscle tissues of MCU^{52} mutants. TUNEL (red), Hoechst (blue).

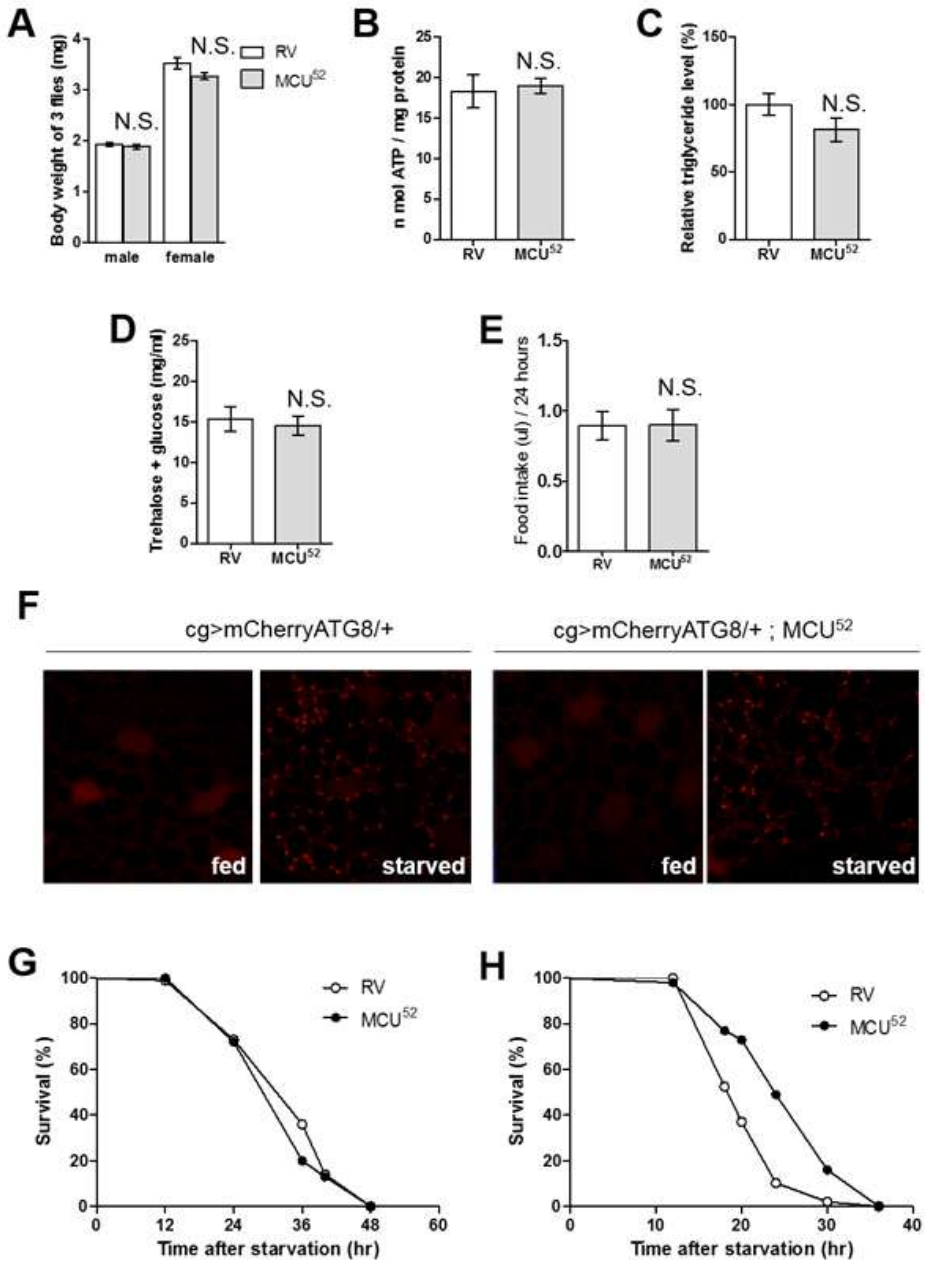

A
Body weight of 3 flies (mg)
N.S.
N.S.
RV
MCU52
male
female
B
n mol ATP / mg protein
N.S.
RV
MCU52
C
Relative triglyceride level (%)
N.S.
RV
MCU52
D
Trehalose + glucose (mg/ml)
N.S.
RV
MCU52
E
Food intake (ul) / 24 hours
N.S.
RV
MCU52
F
cg>mCherryATG8/+
cg>mCherryATG8/+ ; MCU52
fed
starved
fed
starved
G
Survival (%)
RV
MCU52
Time after starvation (hr)
H
Survival (%)
RV
MCU52
Time after starvation (hr)

Figure 10. Metabolic phenotypes of MCU^{52} mutants. (A) Body weight of adult MCU^{52} mutants and the revertants. (B) Thoracic ATP levels of adult MCU^{52} mutants and the revertants. (C) Relative triglyceride levels from adult flies. (D) Circulating sugar levels. (E) Amount of food intake. (F) Starvation-induced autophagy. (G, H) Starvation assay of adult female (G) and male flies (H). n=97~100.

Loss of MCU increased resistance to oxidative stress-induced cell death

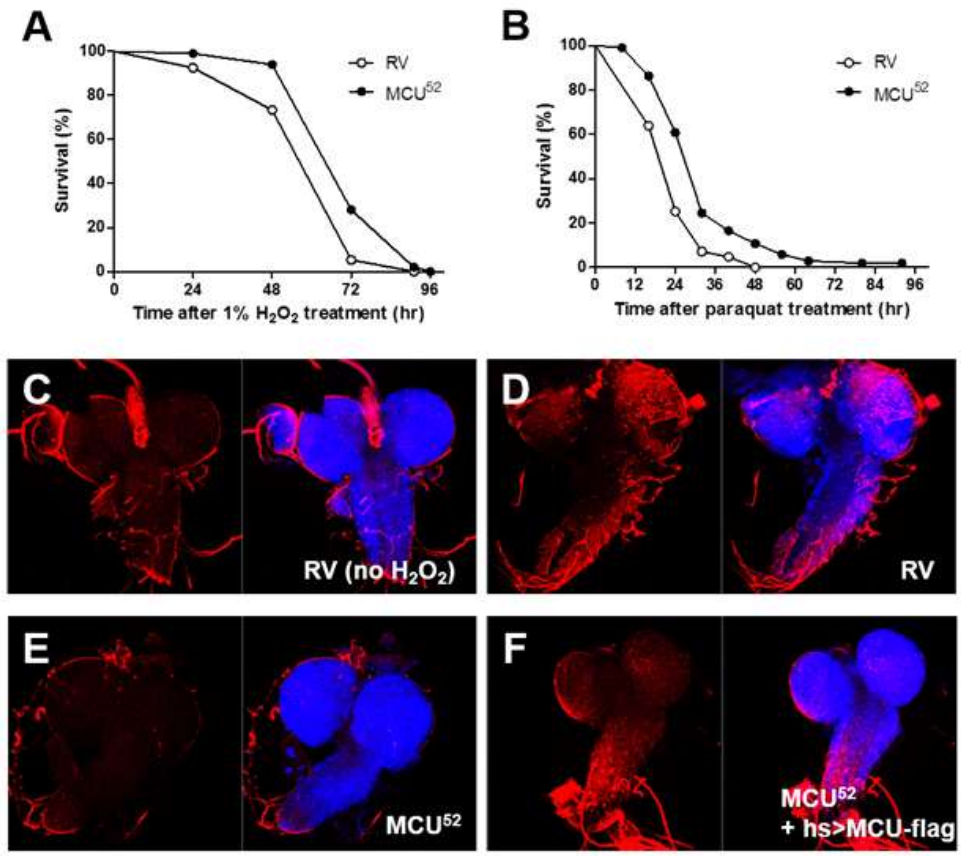
Because oxidative stress increases cytoplasmic and mitochondrial calcium and induces mPTP opening and cell death, we asked if loss of MCU reduces the probability of cell death resulted from mitochondrial calcium overload by preventing aberrant mitochondrial calcium increase in oxidative stress conditions. We investigated survival of MCU^{52} mutants in oxidative stress conditions of H_2O_2 and paraquat feeding. In 1% H_2O_2 food, the survival of MCU^{52} mutants was significantly increased (Fig 11A). Survival of MCU^{52} mutants in paraquat food was also higher than the revertants' (Fig 11B), showing MCU^{52} mutants are more resistant to oxidative stress than the revertants.

To see if oxidative stress-induced cell death is decreased in MCU^{52} mutants, we observed larval brain cell death induced by incubating the larval tissues in 0.1% H_2O_2 solution. In negative control with no H_2O_2 treatment, there was no obvious cell death detected (Fig 11C) while H_2O_2 treatment induced strong cell death signal within the brain tissues of the revertants (Fig 11D). Strikingly, MCU^{52} larval brains showed markedly reduced cell death upon H_2O_2 treatment (Fig 11E). Expressing MCU with hs-gal4 in MCU^{52} mutants increased the cell death signals to some extent (Fig 11F, G), indicating that MCU facilitates cell death induced by oxidative stress. Together, these results suggested that mitochondrial calcium might be involved in oxidative stress-induced cell death and that loss of MCU

might increase resistance to oxidative stress and decrease cell death in oxidative stress conditions.

Increased lifespan in MCU^{52} mutants

Because oxidative stress is involved in aging, I also measured lifespan of MCU^{52} mutants and the revertants. Importantly, MCU^{52} mutants had increased lifespan compared to the revertants (Fig 12). Median survival of MCU^{52} was 10 days longer than that of the revertants.


A
Survival (%)
100
80
60
40
20
0
0
24
48
72
96
Time after 1% H2O2 treatment (hr)
RV
MCU52
B
Survival (%)
0
12
24
36
48
60
72
84
96
Time after paraquat treatment (hr)
RV
MCU52
C
RV (no H2O2)
D
RV
E
MCU52
F
MCU52
+ hs>MCU-flag

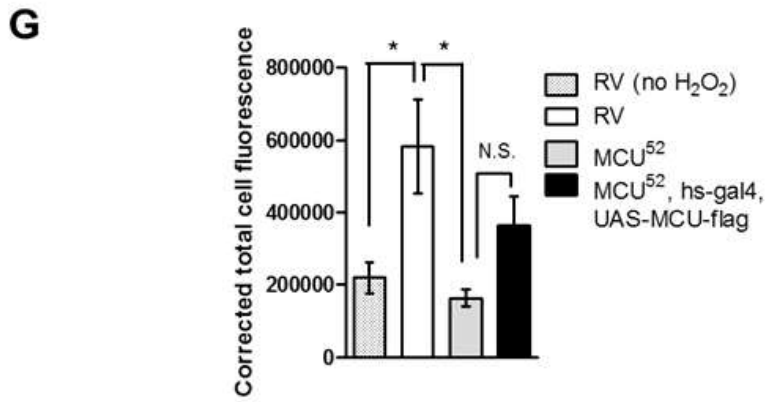

G
Corrected total cell fluorescence
800000
600000
400000
200000
0
*
*
N.S.
RV (no H2O2)
RV
MCU52
MCU52, hs-gal4,
UAS-MCU-flag

Figure 11. MCU^{52} mutants were resistant to oxidative stress than the revertants. (A) Survival of MCU^{52} mutants and the revertants fed with 1% H_2O_2, 5% sucrose. $p < 0.0001$, n=94~100. (B) Survival of MCU^{52} mutants and the revertants fed with 20 mM paraquat, 10% sucrose. $p < 0.0001$, n=83~102. (C-F) H_2O_2-induced cell death of larval brains. (G) Corrected total fluorescence of (C)-(F). Mean ± SEM; $*p < 0.05$, n=2~3.

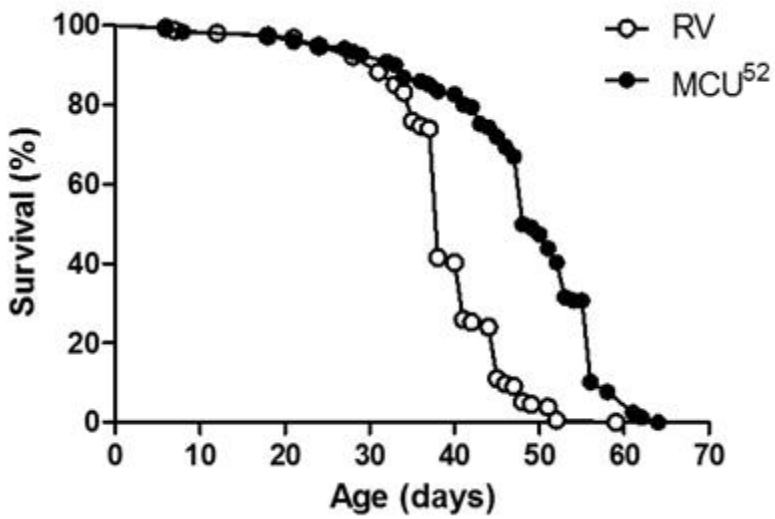


Figure 12. MCU^{52} mutants showed increased lifespan compared to the revertants. Median survival = 38.0 days(RV), 48.0 days(MCU^{52}). n=121~154, $p < 0.0001$.

CONCLUSIONS

In this study, I identified MCU in *Drosophila* and established a *Drosophila* model system to study the roles of MCU in mitochondrial calcium homeostasis in vivo. By generating and charactering MCU loss-of-function mutants, I showed its physiological role and importance in live *Drosophila*. Similar to the mouse loss-of-function mutant, MCU^{52} showed normal development and basal metabolism. MCU^{52} mutants showed mild defects in both spontaneous locomotion (data not shown) and startle-induced locomotion. Reduced climbing ability of MCU^{52} was partially recovered by muscle-specific MCU expression with mef2-gal4. This indicated that loss of MCU resulted in reduced muscle performance as in the loss-of-function mouse model previously reported. Interestingly, significant reduction in spontaneous locomotion of MCU^{52} mutants was restored by hs-gal4-driven MCU expression, but not by mef2-gal4-driven MCU expression. This indicated that reduced movement of MCU^{52} mutants in normal conditions might not be solely dependent on reduced muscle performance. Considering that mitochondrial calcium promotes ATP production and MCU^{52} mutants possess normal metabolic phenotypes, their reduced spontaneous locomotion would compensate for ineffective energy production by reducing energy expenditure.

Importantly, our results suggest MCU lowers the threshold of cell death induced by oxidative stress in vivo. While no other previous studies showed the positive effect of MCU on promoting cell

death upon oxidative stress in vivo, our model proposed MCU as a possible cell death regulator in oxidative conditions. Although some studies using cell culture suggested that MCU might increase cell death by mediating mitochondrial calcium overload, previous in vivo researches on MCU did not show differential cell survival in MCU loss-of-function or gain-of-function mutants.

Deletion of the C-terminal coiled-coil domain or substitution of two acidic residues within the DIME motif failed to generate functional MCU. In previous study, deletion of the C-terminal coiled-coil enhanced calcium current in mitoplast, however our data indicated that this mutant form is nonfunctional uniporter. MCU NIMQ mutant form exerted dominant-negative effect in previous research and our data consistently showed NIMQ mutant is nonfunctional. While other previous researches studied these mutant forms of MCU in the presence of endogeneous MCU, we overexpressed these mutant forms of MCU in the MCU knockout background and measured calcium uptake in vivo, showing a more reliable results about the function of the C-terminal coiled-coil domain and the DIME motif.

We also observed some unexpected phenotypes in MCU loss-of-function mutants. MCU^{52} mutant males showed semi-sterility which is similar to other mitochondrial gene mutants. Lifespan was also increased in MCU^{52} mutants. Considering the role of oxidative stress in aging process, we hypothesized that the increased lifespan of MCU mutants was due to its resistance to oxidative stress. In

starvation assay, survival was significantly increased in male mutants while there was no difference in female mutants. We could not find out which factor caused this sexual difference. A possible explanation is that it is resulted from the physiological difference due to the differential number of sex chromosome genes in males and females.

Although mitochondrial calcium participates in regulation of the enzymes in TCA cycle, cytosolic calcium buffering, cell death, and possibly other unknown processes, our MCU loss-of-function mutants did not exhibit a striking defect but mild phenotypes. In other experiments with our transgenic flies, ectopic expression of MCU in whole body, muscle, neuron, or fat body led to early lethality. MCU overexpression driven by gmr-gal4 resulted in destroyed ommatidia array. These results imply that the absence of rapid mitochondrial calcium uptake does not produce severe disorders but elevated mitochondrial calcium uptake in various tissues is fatal to an organism. Thus, inhibition of mitochondrial calcium uptake may treat disorders resulted from mitochondrial calcium overload with negligible side effects.

Mitochondrial calcium is proposed as a potential target against neurodegenerative diseases, cardiovascular diseases, diabetes, and other mitochondrial diseases (Giorgi et al., 2012). Alteration in mitochondrial calcium homeostasis is an early event in neurodegeneration process. Therefore, targeting mitochondrial calcium might be an effective way to prevent these degenerative diseases. In our study, we found MCU promotes cell death upon oxidative stress

which is known as a critical inducer of neuronal cell death in neurodegenerative disease models. Inhibition of MCU and its related pathways triggering mitochondrial calcium uptake could effectively reduce neuronal and cardiac cell death under pathological conditions.

To summarize, I establishied a *Drosophila* system to study MCU complex genes in vivo. Further studies using *Drosophila* MCU mutants would enable us to discover novel relationship connecting the mitochondrial calcium pathway and other cellular activities. Studying the role of MCU and its related genes will further identify its physiopathological significance in vivo and suggest new strategies to prevent and alleviate mitochondrial diseases.

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국문 초록

초파리에서
MCU 유전자의 기능 연구

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미토콘드리아 칼슘은 다양한 세포 작용에서 중요한 역할을 담당하고 있다. 정상적인 미토콘드리아 칼슘 유입은 에너지 형성을 증대시키고 대사활동을 촉진한다. 그러나 병적인 상황에서는 과도한 미토콘드리아 칼슘 유입, 즉 calcium overload에 의해 세포 사멸을 촉진한다. 따라서 미토콘드리아 칼슘 유입 과정은 미토콘드리아와 세포 항상성을 위해 엄격하게 조절되어야 한다. 최근 이러한 미토콘드리아 내부로의 빠른 칼슘 수송을 담당하는 수송 단백질로 mitochondrial calcium uniporter (MCU)가 규명 되었으며, MCU complex 구성에 참여하는 다른 유전자들도 발견되었다. MCU의 생리학적 기능에 대한 많은 추측이 있으나, in vivo에서 MCU의 기능과 다른 단백질과의 상호작용은 거의 알려져 있지 않다. 이 연구에서 나는 MCU^{52}라고 명명한 MCU 결손 초파리를 제작하여 MCU 발현이 결핍될 경우 미토콘드리아 칼슘 유입이 없음을 보였다. 나는 MCU 돌연변이 초파리의 표현형을 규명함으로써 MCU의 생리학적 기능을 알아보려 하였다. MCU^{52} 돌연변이 초파리는 산화적 스트레스 조건에서 저항성을 보이고 움직임에 이상을 보였으나 그 외 발생, 미토콘

드리아 dynamics, ATP 생성, 그리고 기본 대사 작용은 거의 정상적이었다. 이 연구는 산화적 스트레스에 의한 세포 사멸 과정에서 MCU의 생리학적 역할이 중요할 가능성을 제시한다. 실제 산화적 스트레스 조건에서 MCU^{52} 돌연변이 초파리의 생존이 대조군 초파리에 비해 증가하였다. 이러한 결과와 일관되어 MCU^{52} 돌연변이 larva 조직에서 H_2O_2 처리에 의한 세포 사멸이 감소하였고, MCU를 MCU^{52} 돌연변이 초파리에 다시 발현시킴으로써 이러한 세포 사멸이 증가하였다. 이러한 결과들은 MCU가 산화적 스트레스 상황에서 미토콘드리아 calcium overload에 민감하게 만들고 세포 사멸 유도에 기여함을 보여준다.

주요어: MCU, 미토콘드리아, 미토콘드리아 칼슘, 산화 스트레스
학번: 2013-22950