Regulated EMRE protein level in the Mitochondrial Calcium Uniporter

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ABSTRACT

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A thesis presented to the Department of Biochemistry

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Mitochondrial Calcium Uniporter serves as the major portal for calcium influx into the matrix. It is a highly regulated ion channel complex composed of the pore forming subunit MCU and multiple regulatory subunits, among which the Essential MCU REgulator (EMRE) is necessary for the calcium conducting function of MCU. The goal of this work is to understand how cells regulate EMRE expression level. Using a cycloheximide chase assay, I found that MCU protects EMRE from rapid proteolysis by the mitochondrial m-AAA (ATPases Associated with diverse cellular Activities) protease. Experiments show that such protection requires interactions between the two proteins' transmembrane helices (TMHs). Then I use EMRE homologues and chimeras to show that the rate-limiting step for EMRE degradation occurs after initial protease binding to the EMRE's N-terminus. This work revealed a post-translational

mechanism by which balanced expression of uniporter subunits is achieved, a condition that might be crucial for proper assembly of the uniporter complex.

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List of Abbreviations

MCU= Mitochondria Calcium Uniporter

EMRE= Essential MCU Regulator

TMH= Transmembrane Helix

MICU1=Mitochondria Calcium Uptake 1

MICU2=Mitochondria Calcium Uptake 2

m-AAA=ATPases Associated with diverse cellular Activities protease.

CHX=Cycloheximide

KO=Knock Out

BCA= Bicinchoninic acid assay

RIPA=Radioimmunoprecipitation assay

NBT=nitro-blue tetrazolium

BCIP=5-bromo-4-chloro-3'-indolyphosphate

hEMRE= Human EMRE

Ce EMRE= C.elegans EMRE

Introduction

Calcium in the mitochondrial matrix is crucial for regulating normal cell physiology. (Gunter, Yule et al. 2004, Contreras, Drago et al. 2010, Antony, Paillard et al. 2016) A moderate increase in matrix calcium boosts mitochondrial ATP production, while calcium overload triggering programmed cell death. Steady-state matrix calcium level is governed by constant influx and efflux of calcium across the mitochondrial inner membrane, mediated by a few calcium channels and transporters. Among these transport proteins, the mitochondrial calcium uniporter, a calcium ion channel inhibited by ruthenium red, represents the major calcium entry pathway.(Carafoli 2010, Santo-Domingo and Demaurex 2010). In the past few years, a series of breakthrough demonstrate that the uniporter is a multi-subunit protein complex, composed of the pore-forming subunit MCU and a set of regulatory subunits including EMRE(Essential MCU REgulator), MICU1 (Mitochondria Uptake 1), and MICU2 (Mitochondria Uptake 2).(Perocchi, Gohil et al. 2010, Mallilankaraman, Cardenas et al. 2012, Plovanich, Bogorad et al. 2013, Sancak, Markhard et al. 2013)

This work focuses on the EMRE protein. EMRE is a small (~10 kDa) single membrane-spanning protein located in the inner mitochondrial membrane (IMM). Recent work in my lab (Tsai et al., in press) demonstrates that EMRE serves as a bridge, linking MCU and MICU1 together (Fig. 1a).

For a protein complex, balanced expression of each subunit is crucial. For instance, if EMRE is in molecular excess than MCU and MICU1, a large quantity of EMRE-MICU1 and

EMRE-MCU subcomplexes could form, preventing the formation of full MCU-EMRE-MICU1 complexes. This phenomenon is called “prozone” effect, and its impact could in principle be attenuated if unassembled EMRE is quickly degraded by proteases. Previous work has shown that MCU knock out (KO) leads to drastically reduced EMRE expression in HEK293 cells, implying that these cells might indeed possess a mechanism to ensure that EMRE expression level is close to that of MCU.

This study aims at understanding the mechanism underlying the dependence of EMRE expression level on MCU. By using a cycloheximide chase assay, I found that without MCU, EMRE is degraded quickly, with a short half-life of ~30 min. However, once EMRE binds to MCU, its half-life is increased by >10-fold. Work from my colleagues identifies m−AAA (Tsai et al., manuscript in preparation) proteases being responsible for EMRE degradation. These proteases use the catalytic domain in the matrix to bind the soluble region of a membrane protein, and then utilize the energy of ATP hydrolysis to pull out the protein from membranes for digestion. Using several EMRE homologues and chimeric EMRE, I show that binding of the m−AAA protease to the N-terminus is not the rate-liming step for EMRE degradation. Future work will evaluate the physiological impact if unassembled EMRE is rendered protease-resistant.

Materials and Methods

Cell culture and mammalian cell transfection
HEK 293 cells were grown in a 37℃, 5% CO_2incubator. Transfection is performed with Lipofactamine 3000 reagents.

Cycloheximide (CHX) Chase assay
HEK 293 was cultured in 60 mm dish to 60-70% confluency and transfected with 4.5 ug DNA per dish. After 24hr, cells were split into 4 wells in a 6-well plate. After another 24hr, cells in one of the four wells were harvested as CHX-untreated control (0hr). CHX was then added to the other three wells, at a final concentration of 50ug/ml. These cells were harvested 1, 2 and 4 hrs after CHX addition.

Western Blot
Cells were washed with PBS.100 μL ice cold RIPA buffer was added to each of the 6-wellplate. Lysate was cleared by centrifugation. BCA assay was then used to determine the total protein concentration in the supernatant.20 – 100 ug proteins, depending on the expression level of each protein, were used for standard western analysis. In short, the proteins were transferred to nitrocellulose membranes, which were blocked with milk, incubated with primary antibody, and then an alkaline phosphatase conjugated secondary antibody. Color development is achieved using NBT/BCIP. The 1D4 antibody was a gift from Daniel Oprian's lab, and the EMRE antibody was obtained from Santa Cruz.

Data analysis

Quantification of western-blot band intensity was performed using Image J. Each data point represents at least 3 independent repeats, and is presented as average +/- SEM.

Results

MCU slows down EMRE degradation

It was shown in the literature that MCU-KO reduces EMRE expression, but has no effect on EMRE mRNA level. (Murgia and Rizzuto 2015) This implies that the reduced EMRE expression results from post-translational regulations. To test this idea, I use cycloheximide (CHX), a ribosomal elongation inhibitor, to block protein synthesis, and then follow the time course of EMRE degradation in HEK239 cells. (Garreau de Loubresse, Prokhorova et al. 2014) I found that in MCU-KO HEK293 cells, EMRE has a short lifetime of ~30 min as shown in Fig. 1 b-c. Introducing MCU into these MCU-KO cells greatly prolongs the half-life or EMRE (Fig. 1 b-c). Thus, it appears that MCU could regulate EMRE expression level by protecting EMRE from proteolysis.

MCU binding to EMRE protects EMRE against degradation

How does MCU protect EMRE from being degraded? Since the two proteins directly interact, it is natural to hypothesize that the MCU-EMRE interaction might block protease attacks. I tested this using MCU-EMRE double KO HEK293 cells. I expressed WT-EMRE in the presence or absence of a MCU mutant, A241F, which does not bind EMRE (Tsai et al., in press).The CHX chase assay shows that A241F fails to prolong EMRE lifetime (Fig 2), indicating that the interaction between MCU and EMRE is indeed necessary for MCU to protect EMRE from degradation.

Low steady-state EMRE expression when MCU is incompetent of binding EMRE

As reported in the literature, and as also confirmed by myself (Fig. 3a), MCU-KO essentially eliminates steady-state EMRE expression. My next task is to examine if this phenomenon could be sufficiently explained by a rapid degradation of unassembled, monomeric EMRE, as observed in Fig. 1-2. To test this, I transfected MCU-KO HEK293 cells with either WT or A241F MCU plasmid. Fig. 3b shows that WT MCU restores EMRE expression, while the A241F mutant, which is unable to bind EMRE, failed to increase steady-state EMRE expression. I therefore conclude that selective degradation of uncomplexed EMRE is the major mechanism by which cells synchronize the expression level of MCU and EMRE.

Rate-limiting step of EMRE degradation occurs after protease binding

One of the most crucial issues at this point is how the m-AAA proteases selectively digest the "free" EMRE without its MCU binding partner in the IMM. To address this tough question, it is necessary to understand the kinetics governing molecular events during EMRE degradation. Digestion of EMRE by m-AAA proteases could be roughly divided into two steps. In this first step, the m-AAA protease uses its catalytic domain in the matrix to bind EMRE's N-terminus. In the next step, it uses the energy of ATP hydrolysis to catalyze dislocation of EMRE from the membranes into its central catalytic chamber for proteolysis. I ask which of these two steps is the rate-limiting step for EMRE degradation.

During this study, we accidentally noticed that the *C. elegans* homologue of EMRE (ceERME), which is capable of forming a functional uniporter channel with human MCU, is resistant to degradation by human m-AAA proteases. This provides a tool to probe which components of EMRE has a crucial impact on degradation. Accordingly, I produced EMRE

chimeras with the N-terminal region of hEMRE replaced by the corresponding region from ceEMRE. Interestingly, the mutant is rapidly degraded. In contrast, when the TMH of hEMRE is substituted by the TMH from ceEMRE, the degradation becomes much slower. The results therefore argue that, unexpectedly, the TMH sequence has a large impact on the kinetics of EMRE degradation, suggesting that the rate-limiting step of EMRE degradation occurs after protease binding.

Discussion

The calcium uniporter is the major portal for calcium influx through mitochondrial inner membrane, and is an integral part of the tightly regulated mitochondrial calcium transport system. (Chaudhuri and Clapham 2014).For this multi-subunit channel complex to carry out its function, correct assembly of the subunits is necessary. If EMRE is in large excess than MCU or MICU1, EMRE-MCU and EMRE-MICU1 subcomplexes might form, preventing proper assembly of the full complex. Moreover, it is known that the EMRE-MCU subcomplex is an unregulated, "leaky" calcium channel in the IMM (Tsai et al., in press), whose activity could depolarize the mitochondrial membrane and induce matrix calcium overload, a condition linked to severe neuromuscular disorders in humans. This study shows that cells could achieve balanced MCU and EMRE expression level by selectively eliminating uncomplexed EMRE.

An unresolved issue in this work is how the transmembrane domain interaction between MCU and EMRE slows down EMRE degradation. An obvious possibility is that MCU blocks a protease recognition site in EMRE's N-terminus. If there is such a specific site, it is likely to be eliminated when the N-terminus of hEMRE is replaced by the corresponding region in ceEMRE, as the N-termini in these two homologues have low sequence similarity (Fig. 4b). However, such a chimera is still degraded rapidly (Fig. 4c-d), suggesting that protease-recognition of EMRE's N-terminus is not the rate-limiting step for degradation. I recognize an alternative possibility that the N-terminus of ceEMRE might still possess a sequence recognizable by the m-AAA protease.

To rule out the presence of such specific m-AAA protease site, it will be crucial in the future to scramble the N-terminal sequence of EMRE, and determine the rate of degradation.

The results from the chimera study also show that the TMH sequence in EMRE has a great impact of the rate of degradation. In a sense, this is not surprising, as it is known from the excellent work of Bob Sauyer that AAA proteases repeatedly hydrolyze ATP, until one ATP hydrolysis event eventually unfolds the protein. It's conceivable that if EMRE interacts with lipid more tightly, it will require more ATP hydrolysis attempts for the m-AAA protease to successfully extract the protein from the membrane for digestion. It follows that MCU binding might slow down the degradation of EMRE by providing additional binding energy that m-AAA protease must overcome, by more events of ATP hydrolysis, before proteolysis could occur. This hypothesis could be tested in the future by using atomic force microscopy to quantify the force required to extract EMRE from the liposome membrane in the presence or absence of MCU.

Figures

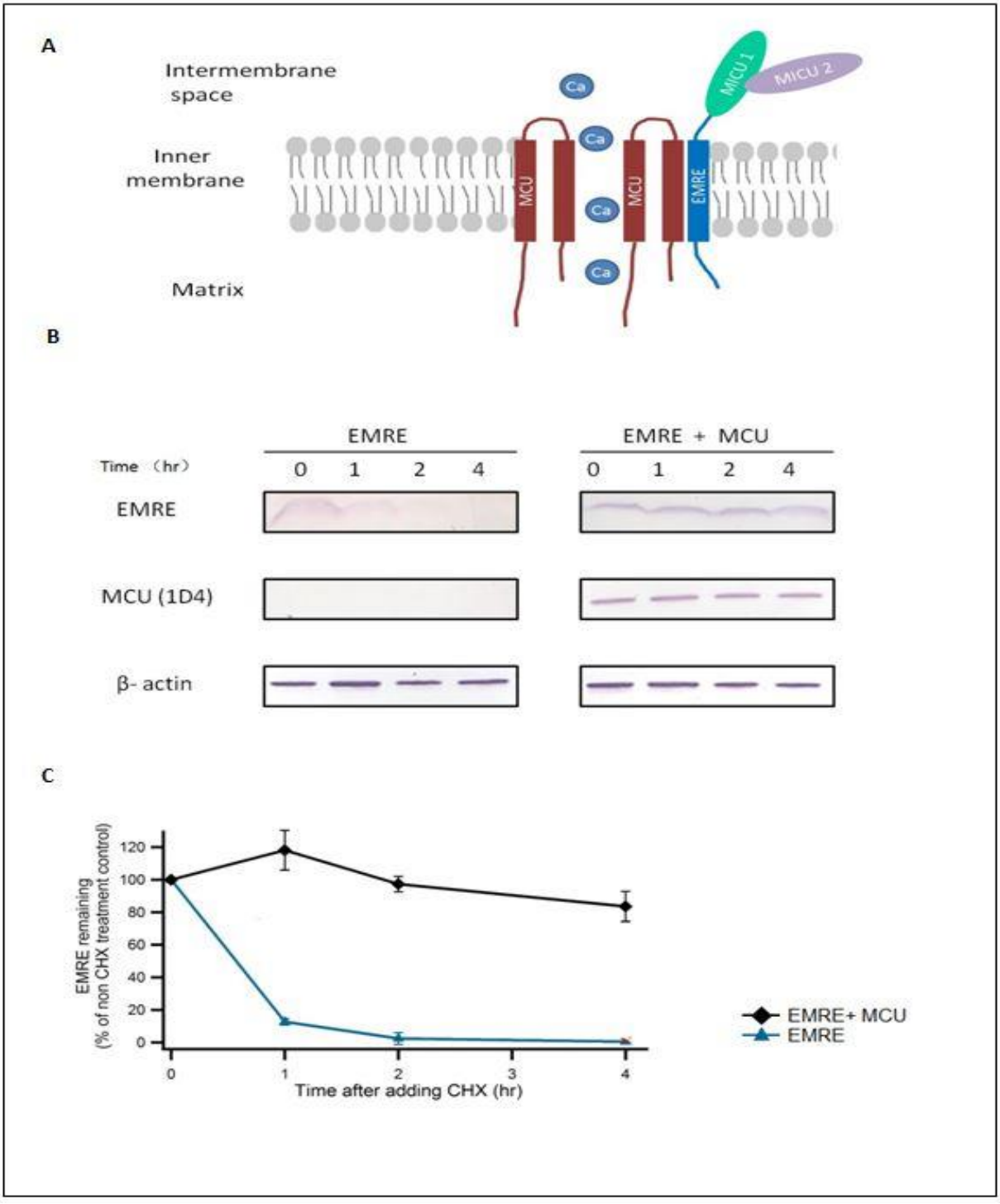
A
Intermembrane space
Inner membrane
Matrix
Ca
MCU
MCU
EMRE
MICU 1
MICU 2
B
EMRE
EMRE + MCU
Time （hr）
0 1 2 4
0 1 2 4
EMRE
MCU (1D4)
β- actin
C
EMRE remaining (% of non CHX treatment control)
120
100
80
60
40
20
0
0 1 2 3 4
Time after adding CHX (hr)
EMRE+ MCU
EMRE

Figure 1 MCU slows down EMRE degradation

A) Illustration of the uniporter complex

B) Western blot showing the EMRE protein amount with or without MCU after 1hr,2hr and 4 hrs CHX treatment, the untreated samples as control （0 hr）

C) Line chart made from three independent experiments, presented as average +/- standard error.

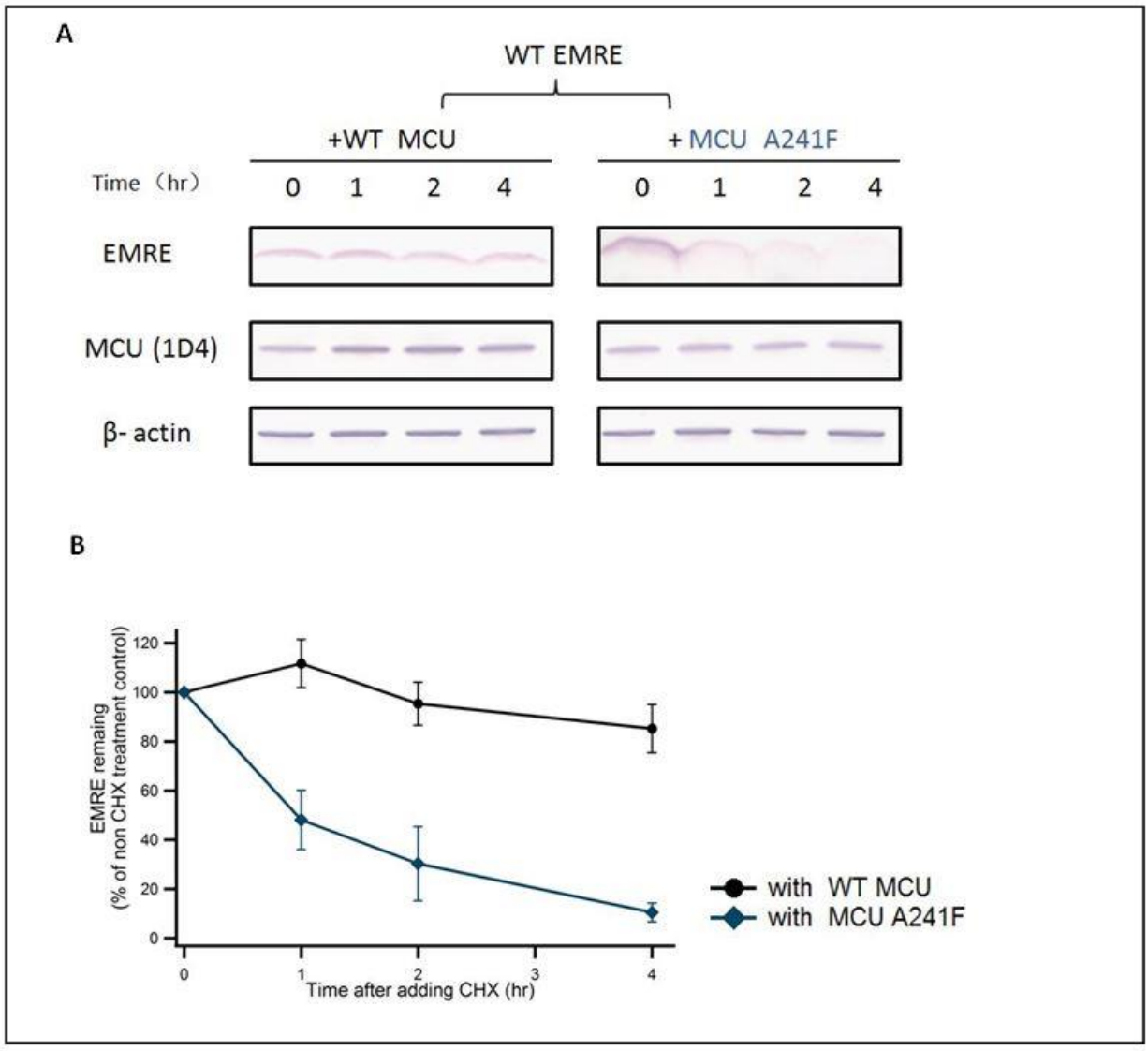

Figure 2 EMRE degradation profile, when co expressed with MCU or MCU A241F

A) Western blot showing the EMRE protein level when co expressed with MCU or MCU A241F after 1hr,2hr and 4 hrs CHX treatment, the untreated sample as control (0 hr)

B) Line chart made from three independent experiments, presented as average +/- standard error.

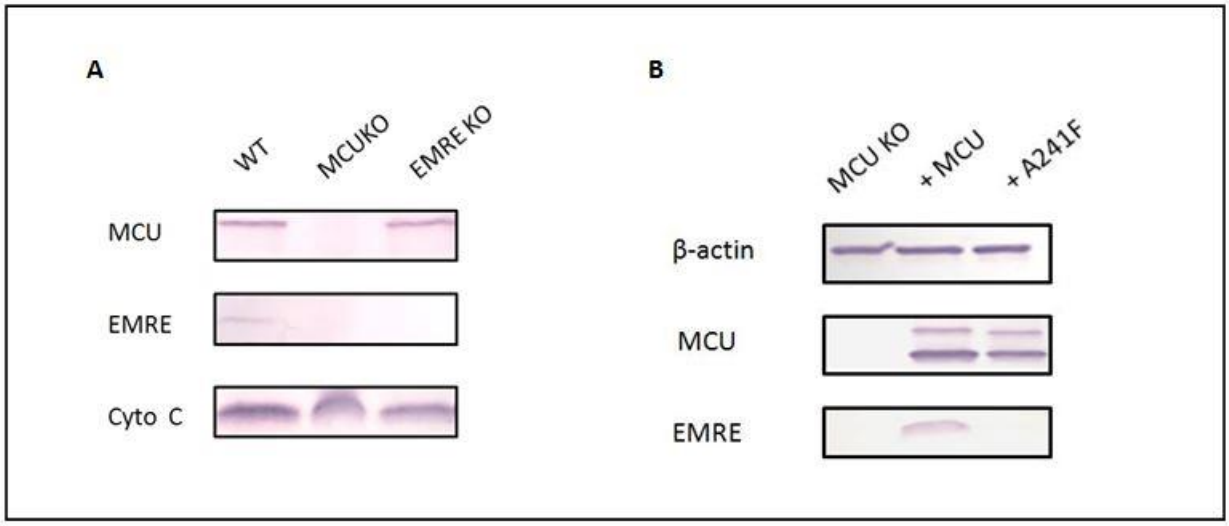

Figure 3Steady state EMRE protein amount with or without the presence of binding competent MCU

A) Western blot showing MCU, EMRE amount in WT, MCUKO and EMRE KO cells, Cytochrome C as loading control.

B) Western blot showing EMRE level in MCU KO cells when co expressed with MCU or MCU A241F, MCU KO as negative control, β-actin as loading control.

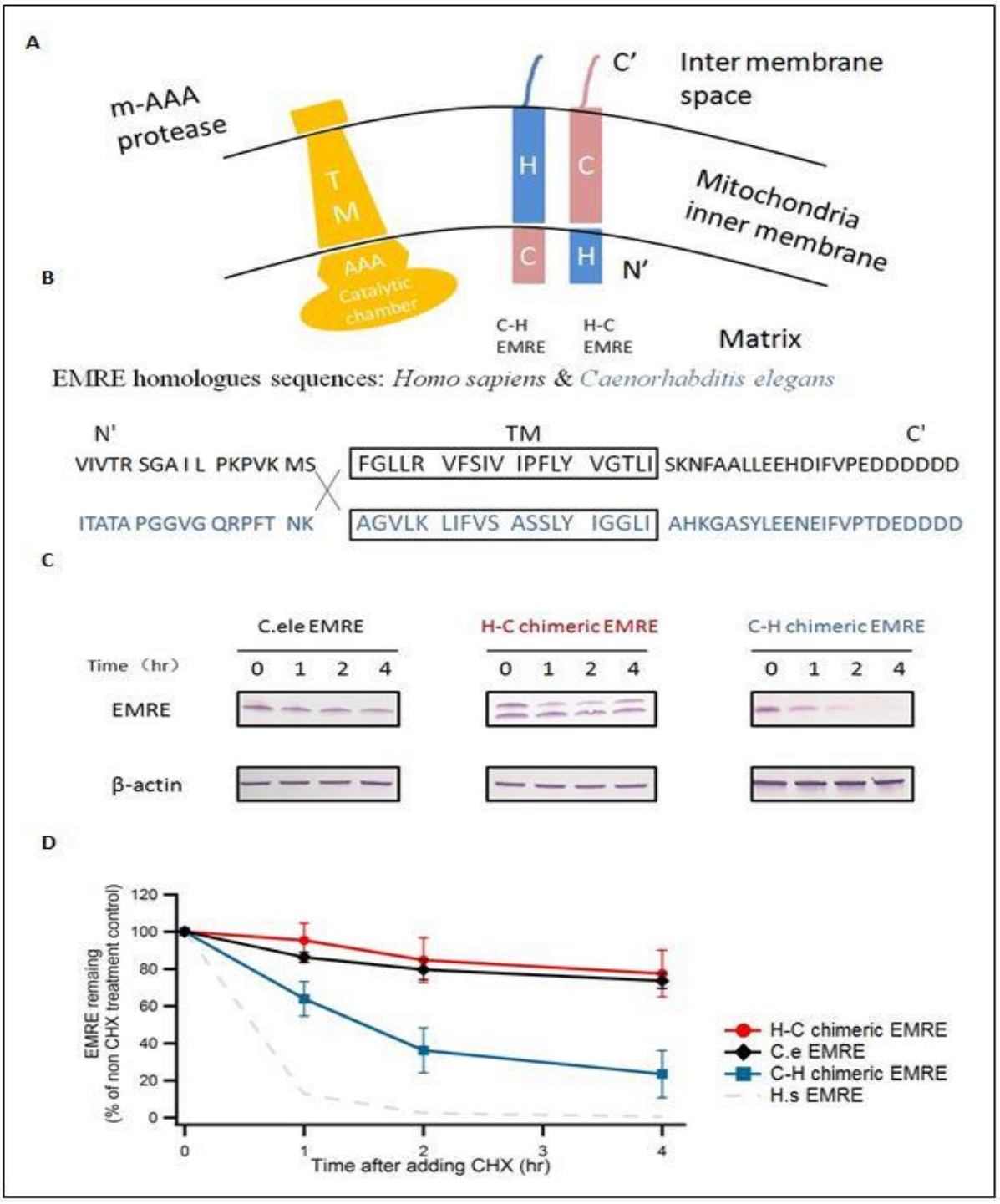

Figure 4C.elegans EMRE, chimeric EMRE degradation profile.

A) Illustration of m-AAA protease and two chimeric EMRE constructs. Topology and domain composition were demonstrated.

B) Sequence of human and C.elegans ERME. Transmembrane domain is indicated.

C) Western blot showing the different EMRE constructs' amount after 1hr,2hr and 4 hrs CHX treatment, the untreated sample as control (0 hr).

D) Line chart made from three independent experiment results, presented as average +/- standard error.

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