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이학석사학위논문

미토콘드리아 내막 단백질 삽입에  
관여하는 TIM23 복합체의 구성요소  
연구

Investigation of components of the TIM23  
complex involved in lateral insertion of  
mitochondrial inner membrane proteins

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유수지

Investigation of components of the  
TIM23 complex involved in lateral  
insertion of mitochondrial inner  
membrane proteins

by

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# ABSTRACT

Mitochondrial inner membrane (IM) proteins carrying a cleavable N-terminal presequence are encoded by nucleus and inserted into the IM by translocase of outer membrane (TOM complex) and translocase of inner membrane (TIM23 complex). In addition, the TIM23 complex mediates translocation of proteins into the matrix. How the TIM23 complex recognizes different localization signals of precursor proteins and sorts into the IM or matrix remains elusive. To better understand the mechanisms of the TIM23-mediated membrane protein insertion in mitochondria, I undertook two approaches. First, I attempted to identify the regions or residues in the TIM23 components critical for lateral insertion of proteins into the IM by genetic screening. This approach identified Tim17 mutant that allows enhanced membrane insertion of a hydrophobic segment containing Proline residue that normally translocates to the matrix. Sequencing analysis shows that this Tim17 mutant is truncated at the C-terminus, lacking the loop region in the matrix. Since this region is shown important for interaction with a motor component in the matrix, it is speculated that impaired interaction between the TIM23 complex and the matrix motor complex might cause decreased translocation efficiency of proteins into the matrix.

Second approach focused on Mgr2 which is a newly identified subunit of the TIM23 complex. When Mgr2 is absent, interaction of the TIM23 core with Tim21 and respiratory chain complexes are shown impaired. Mgr2 is also shown to be involved in the TOM-TIM23 cooperation and protein import to the matrix. Another study revealed that membrane protein insertion efficiency was increased in the absence of Mgr2 whereas overexpression of Mgr2 delayed the insertion, so they reported Mgr2 as a lateral gatekeeper. To investigate whether Mgr2 has a general role in regulating lateral insertion of proteins, membrane insertion efficiency of Mgm1 variants with diverse sequence characteristics were determined in Mgr2 mutants. The results show that overexpression of Mgr2 increases hydrophobicity threshold for membrane insertion of a transmembrane domain whereas a lack of Mgr2 decreases. Mgm1 variants carrying alterations in flanking charged residues of the transmembrane domain were better inserted when Mgr2 was deleted. Insertion of a transmembrane domain containing Proline residues was decreased in the absence of Mgr2. So far these data suggest that Mgr2 is involved in sensing the determining features of the transmembrane domain and has a role in modulating membrane protein insertion.

Key words: TIM23 complex, lateral insertion, Tim17, Mgr2, Tim23, membrane proteins, transmembrane domain, Mgm1, mitochondrial protein sorting

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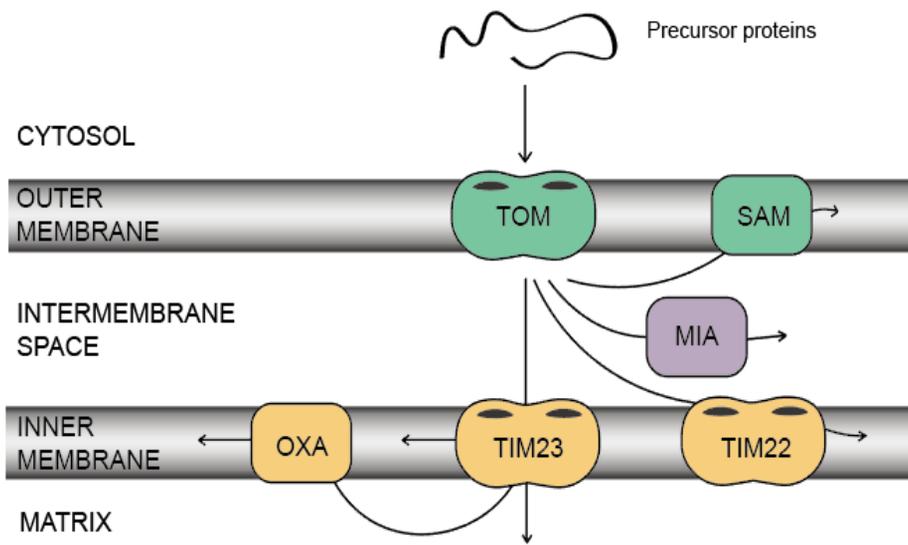
# INTRODUCTION

Mitochondria are vital cell organelles involved in numbers of cellular processes including energy metabolism, signaling, apoptosis, and biosynthesis of Fe-S clusters, urea, lipids, and amino acids [1] [2]. The complex structure of mitochondria reflects the diversity of mitochondrial functions. Mitochondria consist of two membranes, the outer membrane (OM) and the highly folded inner membrane (IM), and two aqueous compartments, the intermembrane space (IMS) and the innermost matrix.

## ***Mitochondrial translocases and protein sorting***

About 1000 mitochondrial proteins are encoded in the nucleus and synthesized as precursor in the cytosol, whereas eight are encoded in the mitochondrial DNA in *Saccharomyces cerevisiae* [3] [4]. Mitochondrial precursor proteins in cytosol are subsequently targeted into proper mitochondrial subcompartments by several complex machineries called translocases (Figure 1). Majority of mitochondrial proteins firstly go through the translocase of the outer membrane (TOM) [5]. The TOM complex and the sorting and assembly machinery (SAM) mediate the insertion of OM proteins [6]. IMS proteins are sorted by either mitochondrial intermembrane space assembly (MIA) or the translocase of the inner membrane (TIM23). MIA and small Tim proteins aid oxidative folding and translocation of IMS proteins with cysteine motif [7]. In another case, proteins are targeted to the TIM23 complex and cleaved sequentially by matrix processing peptidase (MPP) and IM peptidase (IMP), and mature proteins are released to IMS [8]. For IM proteins, TIM22, TIM23, and the oxidase assembly 1 (OXA1) are involved in the insertion. While carrier proteins containing internal sorting signals are

inserted into the IM by the TIM22 complex, most of IM proteins carry the cleavable N-terminal presequence and are targeted to the TIM23 complex [9]. After the presequence is cleaved by MPP, proteins take either the stop transfer or the conservative sorting pathway for insertion into the IM [10]. Proteins with sorting signals in a transmembrane (TM) segment are arrested at TIM23 and laterally released into the IM – stop transfer pathway [11]. On the other hand, proteins following the conservative sorting pathway are initially fully translocated to the matrix and then inserted into the IM from the matrix with the help of the OXA1 [3]. The OXA1 is also involved in the insertion of IM proteins encoded by the mitochondrial DNA.



**Figure 1. Sorting of mitochondrial proteins**

The translocase of the outer membrane (TOM) is the common entry gate for mitochondrial proteins. Precursor proteins in the cytosol are imported and sorted into different mitochondrial subcompartments, which are outer membrane, intermembrane space, inner membrane, or matrix. SAM, the sorting and assembly machinery; MIA, mitochondrial intermembrane space assembly; TIM22 and TIM23, translocase of the inner membrane; OXA, insertase of the inner membrane

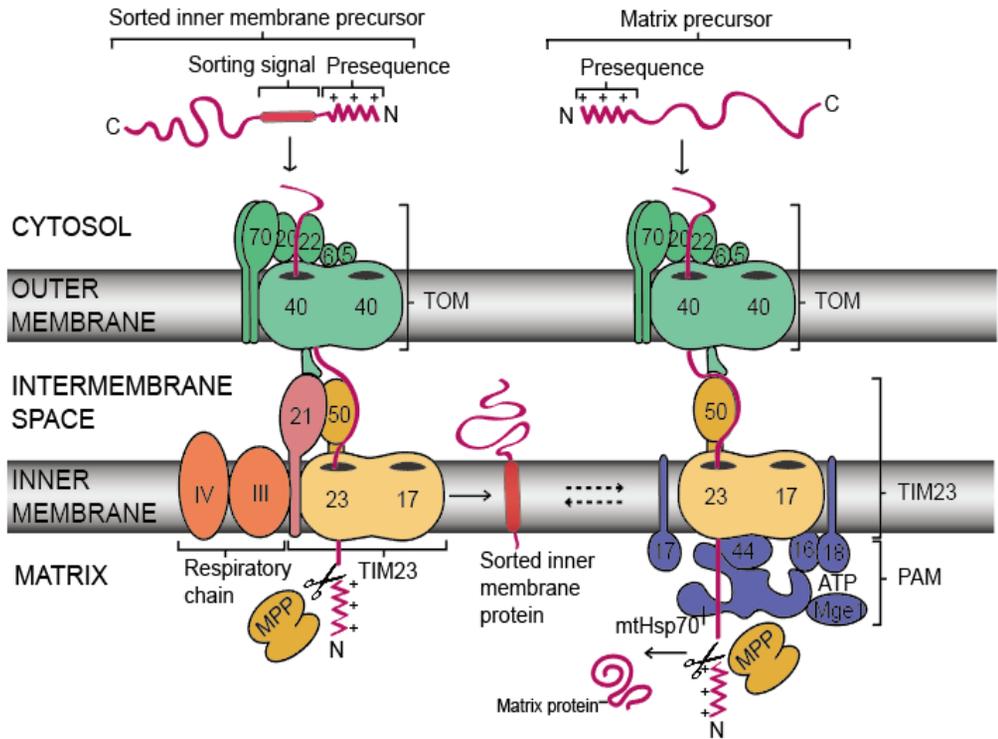
### ***TIM23 complex***

The TIM23 complex is capable of directing presequence carrying mitochondrial proteins across as well as into the IM. It transports precursor proteins into two different subcompartments, the matrix and the IM. Tim50 acts as a receptor that recognizes presequence of proteins [12-15]. In a membrane potential dependent manner, proteins are then transferred to the channel formed by Tim23 and possibly also Tim17 and/or Mgr2 (Figure 2, Mgr2 not shown). For insertion of IM proteins, Tim21 along with Mgr2 associates the core subunits of the TIM23 complex (Tim23, Tim17, and Tim50) with the respiratory chain complex [17]. In contrast, the presequence translocase associated import motor (PAM) including mtHsp70, Pam16, Pam18 and Mge1 is recruited to the TIM23 via Tim44 and performs complete translocation for sorting of matrix proteins [18-20]. Depending on the destination of the substrate, the complex formation is dynamically regulated. Nevertheless, the mechanism of channel opening for lateral insertion of IM proteins remains largely unknown. Further studies on domains of Tim23/Tim17 or other subunits responsible for lateral insertion, such as recognition of proteins, regulating channel opening, and interaction with other subunits of the TIM23 complex are in demand.

### ***Determinants of mitochondrial IM protein insertion***

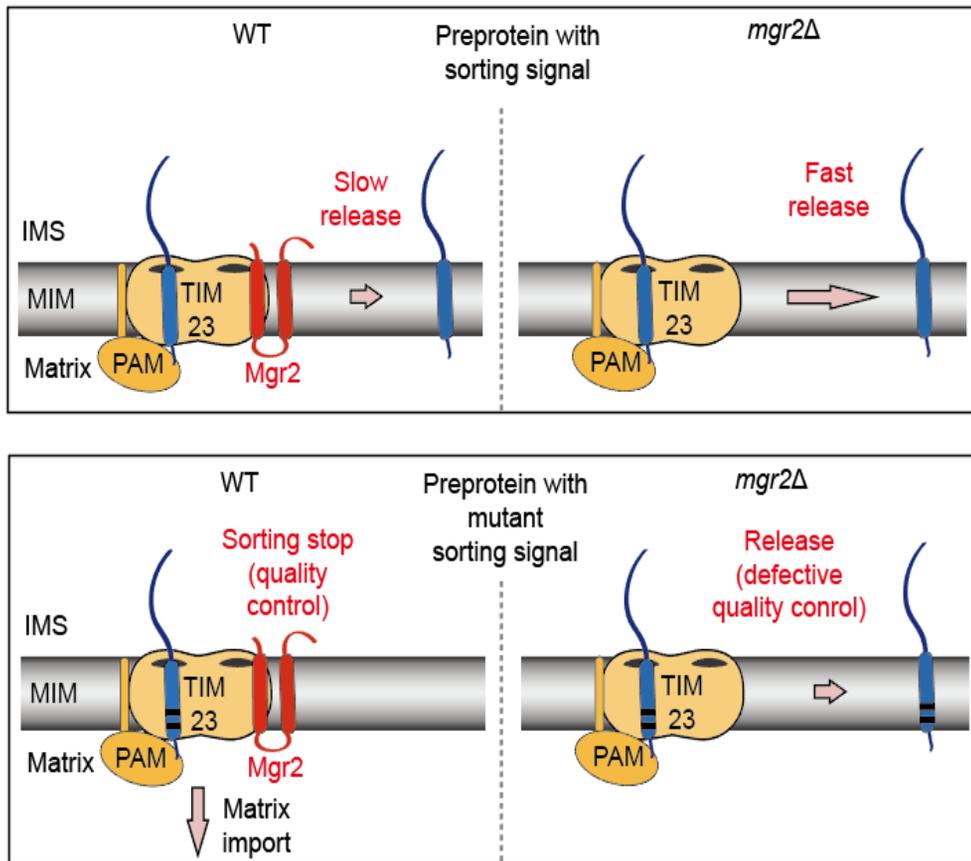
The majority of integral membrane proteins have tightly packed  $\alpha$ -helical structure, and overall hydrophobicity has been recognized as the main characteristic of the transmembrane (TM)  $\alpha$ -helices for translocase-mediated recognition and insertion into the lipid bilayer [21]. The biological scale is reported for the TIM23-mediated membrane protein insertion into the IM, and there is a strong correlation with that of the endoplasmic reticulum (ER), with

leucine being the most hydrophobic and arginine being the most hydrophilic [21]. In addition to hydrophobicity of the transmembrane domain (TMD), the presence of a proline residue in the TMD and charged residues flanking the TMD are also known as determinants of membrane protein insertion [10] [22]. In the mitochondrial IM, the hydrophobicity required for retention of a TMD containing alanine and leucine stretch is affected by mAAA protease which actively dislocates a TMD from the IM [23]. It might contribute to the differences in threshold hydrophobicity and effects of charged residues flanking the TMD observed between mitochondrial IM and ER membrane. However, the components responsible for the difference in TMD recognition or defining hydrophobicity required for mitochondrial IM protein insertion at the level of TIM23 remained unknown. Mgr2 was recently reported to delay the insertion of model IM proteins, Cytochrome b2-DHFR (Cyb2-DHFR) and Mgm1 [16]. Besides, in the absence of Mgr2, a protein lacking the sorting signal was inserted to the IM (Figure 3). Thus, Mgr2 was proposed as a lateral gatekeeper of the TIM23 complex, and it gives a question whether Mgr2 acts generally on membrane protein insertion into mitochondrial IM and involved in screening of TMD for the insertion.



**Figure 2. Import pathway of mitochondrial proteins carrying the presequence**

The precursor proteins are imported by the translocase of the outer membrane (TOM) complex, which involves recognition by the receptors Tom20 and Tom22 and translocation through the Tom40 channel. The translocase of the inner membrane complex, TIM23, functions in two modular forms. The TIM23 complex additionally contains Tim21, which transiently binds to the TOM complex and a supercomplex of the respiratory chain (left). In this case, the TIM23 complex mediates the lateral release of proteins with hydrophobic sorting signals into the inner membrane. The other TIM23 form lacks Tim21 but interacts with presequence translocase-associated motor (PAM) and transports proteins into the matrix (right). The membrane potential drives translocation of the presequence through the TIM23 complex and ATP powers the chaperone mtHsp70 (matrix 70 kDa heat shock protein) of the PAM. Mitochondrial processing peptidase (MPP) removes the presequence.



**Figure 3. The schematic diagram of mitochondrial IM protein insertion in the presence or absence of Mgr2.**

If Mgr2 acts as a lateral gatekeeper, the presence of Mgr2 would delay the insertion of membrane proteins and the absence of Mgr2 would favor the insertion of proteins into the membrane even for the protein with impaired sorting signal.

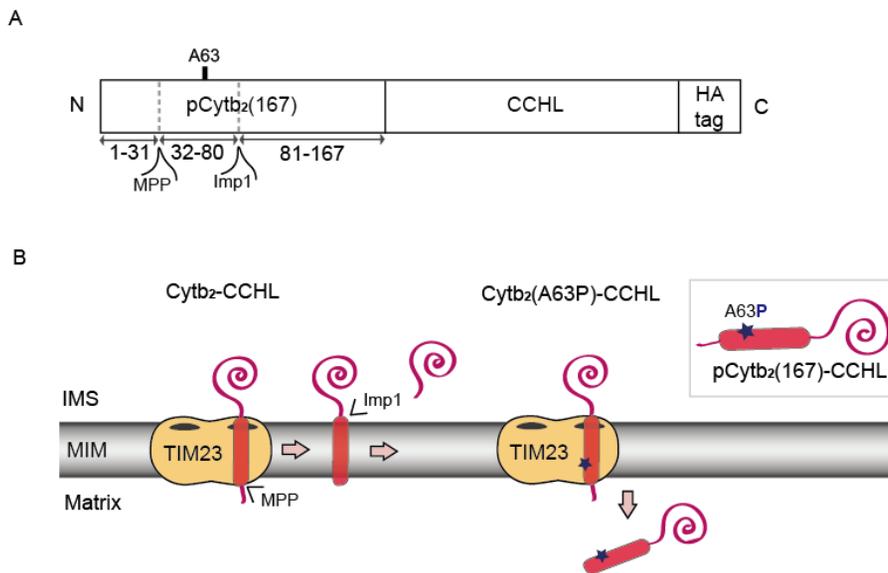
## ***Experimental approaches***

### I. Screening for Tim23/Tim17 mutants with enhanced proline tolerance

To identify the domain of Tim23/Tim17 involved in insertion of a TMD, random mutagenesis screening was designed. The selection scheme utilizes a  $\Delta cyc3$  strain and a model fusion protein made of Cytb<sub>2</sub> and cytochrome c heme lyase (CCHL). Cytb<sub>2</sub> is an IMS protein. It is first targeted to the matrix via its N-terminal presequence which is cleaved by MPP, and becomes i-Cytb<sub>2</sub>. i-Cytb<sub>2</sub> is then inserted into the IM and cleaved again by Imp1, releasing m-Cytb<sub>2</sub> into the IMS [24]. However, when alanine 63 (A63) is mutated to proline residue (P), Cytb<sub>2</sub> is translocated to the matrix instead of sorting to the IM (Figure 4). By using this differential targeting of Cytb<sub>2</sub>, mature CCHL was fused to wild type preCytb<sub>2</sub>(167) and preCytb<sub>2</sub>(167) with A63P mutation. Whereas pCytb<sub>2</sub>(167)-CCHL is inserted into the IM, pCytb<sub>2</sub>(A63P, 167)-CCHL is translocated to the matrix. CCHL is encoded by Cyc3 gene and it is required at the IMS for cell survival on non-fermentable carbon source like YPG [25]. Therefore, when  $\Delta cyc3$  strain has expressed pCytb<sub>2</sub>(167)-CCHL, it can grow on non-fermentable carbon source, whereas expression of pCytb<sub>2</sub>(A63P, 167) cannot rescue the growth defect.

$\Delta cyc3$  strain with pCytb<sub>2</sub>(A63P, 167)-CCHL cannot survive on non-fermentable carbon source. However, if this protein could be inserted into the IM and finally released to the IMS, it would rescue the growth defect on non-fermentable carbon source. Insertion of Cytb<sub>2</sub> is mediated by the TIM23 complex. As Tim23 and Tim17 are known to form a channel of the TIM23 complex, Tim23 and Tim17 were randomly mutagenized by error-prone PCR and expressed in  $\Delta cyc3$  with pCytb<sub>2</sub>(A63P, 167)-CCHL. The aim of Tim23/Tim17 screening using this selection scheme is to find mutant tim23/tim17 with enhanced efficiency for inserting the model protein pCytb<sub>2</sub>(A63P, 167)-CCHL, that is, with enhanced proline tolerance. After selection on non-fermentable carbon source, any viable colony is a probable

candidate responsible for enhanced membrane insertion by the TIM23 (Figure 5). The *tim23/tim17* mutant capable of supporting the cell growth of  $\Delta cyc3$  strain expressing pCytb2(A63P, 167)-CCHL will be confirmed by Western blotting, isolated, and sequenced. Additionally, identified mutants will be retransformed into  $\Delta cyc3$  strain with the model protein or other proteins to validate enhanced membrane protein insertion efficiency.



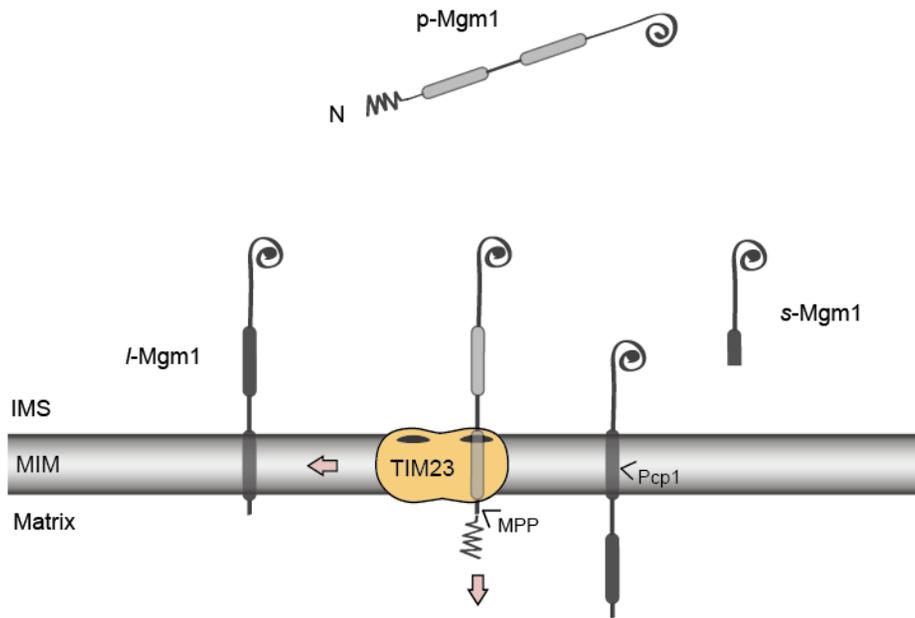
**Figure 4. The sorting of pCytb<sub>2</sub>-CCHL and pCytb<sub>2</sub>(A63P)-CCHL.**

(A) The schematic diagram of pCytb<sub>2</sub>(A63P)-CCHL. (B) Cytb<sub>2</sub> is sorted into the mitochondrial IM by the TIM23. However, mutation from alanine to proline at position 63 causes the protein to be translocated into the matrix.



## II. Examination of the role of Mgr2 in membrane protein insertion via the TIM23 complex

To study whether Mgr2 regulates membrane protein insertion into the mitochondrial IM, Mgr2 deletion ( $\Delta mgr2$ ), Mgr2 overexpression (Mgr2 $\uparrow$ ) and the corresponding wild type (WT) (YPH499) strains were acquired from Dr. Pfanner [17,16]. For comparison of membrane protein insertion in these three strains, Mgm1-based proteins were used for model substrates. Mgm1 has alternative topogenesis, naturally generating two forms, *l*-Mgm1 and *s*-Mgm1 [26]. Mgm1 has two TMD and when the 1<sup>st</sup> TMD is inserted into the IM, *l*-Mgm1 is generated. *s*-Mgm1 is generated when the 1<sup>st</sup> TMD is translocated across the IM, while the 2<sup>nd</sup> TM is inserted into the membrane and exposes a rhomboid cleavage site that is processed by Pcp1. This cleavage results in the generation of *s*-Mgm1 in the intermembrane space (IMS) (Figure 6). In wild type cells, there are roughly the same amount of *l*-Mgm1 and *s*-Mgm1.



**Figure 6. The schematic diagram of Mgm1 topogenesis.**

Mgm1 contains a presequence, two TMDs, and a soluble C-terminal domain. The 1<sup>st</sup> TMD of Mgm1 is sorted into the IM by the TIM23 complex to generate *l*-Mgm1 or translocated across the membrane to generate *s*-Mgm1.

By taking advantage of Mgm1 with two forms, the effect of Mgr2 was tested for determining features of membrane protein insertion. *l*-Mgm1 and *s*-Mgm1 represents membrane inserted and translocated Mgm1 respectively, thus the ratio of two forms was an indicator of membrane protein insertion efficiency. Firstly, the effect of Mgr2 deletion or overexpression on the hydrophobicity required for membrane protein insertion was examined. Mgm1 variants, Mgm1\_A/Ls containing replaced 1<sup>st</sup> TMD with 19 amino acid stretch composed of *n* leucines and *19-n* alanines were used [21]. The more the number of leucine is, the more hydrophobic the 1<sup>st</sup> TMD is. For analyzing membrane insertion efficiency of a set of Mgm1\_A/Ls in WT,  $\Delta mgr2$ , and Mgr2 $\uparrow$ , the ration of *l*-Mgm1 and *s*-Mgm1 was compared.

Subsequently, the same screening was done with other sets of Mgm1 variants. Based on wild type Mgm1, some charged residues flanking the 1<sup>st</sup> TMD of Mgm1 were exchanged to alanine residues. In addition, positively charged lysine (K) or negatively charged aspartic acid (D) is added in the upstream or the downstream of the 1<sup>st</sup> TMD of Mgm1\_A/L, respectively. Using flanking charged Mgm1 variants, the insertion of these charged mutant proteins in Mgr2 deletion or overexpression strains were investigated. Moreover, another set of Mgm1 variants that some amino acids in the 1<sup>st</sup> TMD of wild type Mgm1 were mutated to proline residues are monitored as the same way. Mgm1 variants which contains the alanine and leucine stretch with two prolines in the 1<sup>st</sup> TMD of Mgm1 are also investigated.

# MATERIALS AND METHODS

## ***Yeast strains***

W303-1 $\alpha$  (*MAT $\alpha$* , *ade2*, *can1*, *his3*, *leu2*, *trp1*, *ura3*), BY4741 (*MAT $\alpha$* , *his3*, *leu2*, *met15*, *ura3*),  $\Delta$ *cyc3* (BY4741, *cyc3::KANMX6*),  $\Delta$ *cyc3* $\Delta$ *tim23* (W303-1 $\alpha$ , *cyc3::KANMX6*, *tim23::HIS3* [pRS316-TIM23]),  $\Delta$ *tim17* (W303-1 $\alpha$ , *tim17::HIS3* [pRS416-TIM17]), YPH499 (*MAT $\alpha$* , *ade2*, *lys2*, *his3*, *leu2*, *trp1*, *ura3*),  $\Delta$ *mgr2* (YPH499, *mgr2::KANMX6*), Mgr2 $\uparrow$  ( $\Delta$ *mgr2* [pPGK-MGR2]) [16], temperature sensitive *pam16-3* mutant and the isogenic PAM16 wild type strain [27].

## ***Plasmids and Yeast cell culture***

For the TIM23 random mutagenesis study, pHP84 vectors encoding CYC3, CYC3-HA, pCytb<sub>2</sub>-CCHL-HA or pCytb<sub>2</sub>(A63P)-CCHL-HA were transformed into BY4741 and  $\Delta$ *cyc3* strains. The transformants were selected on –Leu at 30°C and streaked on YPG at 30°C for the growth assay. For the Mgr2 study, pHP84HA vectors encoding Mgm1 or Mgm1 variants were transformed into YPH499,  $\Delta$ *mgr2* and Mgr2 $\uparrow$  strains for analyzing the effect of Mgr2. The transformants were selected on –Leu or –Leu-Ura plate at 30°C.

## ***Random mutagenesis approach***

For screening for the TIM23 complex with enhanced protein insertion efficiency, Tim23 and Tim17 were randomly mutagenized by using error-prone PCR. Generated mutants were then inserted into p426GPD vector for overexpression by homologous recombination. Mutated Tim23 or Tim17 in

p426GPD vector was co-transformed with pCytb<sub>2</sub>(A63P)-CCHL into  $\Delta cyc3$  strain, and then selected colonies on –Leu were transferred to YPG plate by replica plating. Survived colonies on YPG are regarded as candidates with enhanced protein insertion, thus validated. For validation of candidate mutants, processing of pCytb<sub>2</sub>(A63P)-CCHL was monitored by Western blotting.

### ***Protein preparation, SDS-PAGE, and Western blotting***

After growing yeast transformants at 30°C overnight, proteins were prepared using TCA (Trichloroacetic acid) precipitation or crude membrane fractionation. In TIM23 random mutagenesis study, cells were grown until reaching OD<sub>600</sub> 0.8-1.0 and then 15 OD<sub>600</sub> units of cells were washed and lysed with small beads and ice-cold lysis buffer. Non-lysed cells were removed and proteins were precipitated as the pellet after centrifugation at 4°C for 30 minutes - crude membrane fractionation. Afterwards, SDS-PAGE and Western blotting with an anti-HA antibody were followed. Western blots were developed with Amersham Bioscience Advanced ECL kit on a Biorad Chemi-doc-XRS+ system (Biorad) [36]. In Mgr2 study, 1 OD<sub>600</sub> unit of cells were washed, incubated at 4°C for 10 minutes with alkaline mix (185mM NaOH, 10mM phenylmethylsulfonyl fluoride (PMSF),  $\beta$ -mercaptoethanol), and incubated again at 4°C for about 20 minutes after mixed with TCA (50%). After two steps of cold centrifugation, proteins were precipitated – TCA precipitation. SDS-PAGE and Western blotting were followed as above.

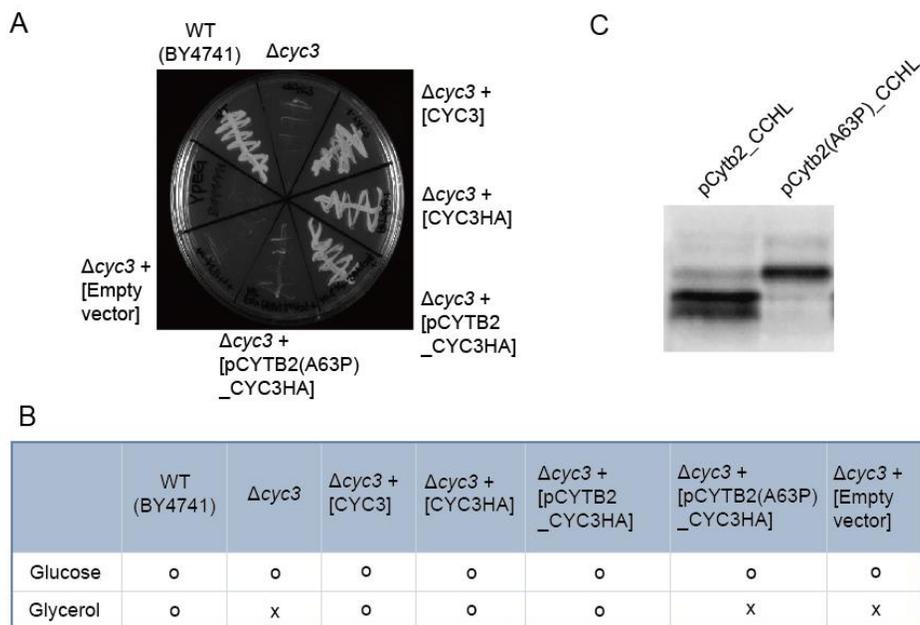
## RESULTS

### *Selection scheme for Tim23/Tim17 screening is validated*

To better understand the mechanisms of the TIM23 complex-mediated membrane protein insertion in mitochondria, genetic screening was undertaken. The aim of this approach is to find regions of TIM23 components critical in lateral insertion of a hydrophobic segment containing proline residue. Introduction of proline to a transmembrane domain (TMD) of mitochondrial IM proteins reduces efficiency of membrane insertion by the stop-transfer pathway. As Tim23 and Tim17 are central subunits of the TIM23 complex involved in channel formation, they were randomly mutagenized. Screening by selection scheme was done to select Tim23 or Tim17 mutant which allows enhanced membrane insertion activity of proteins containing proline residue.

The selection scheme for random mutagenesis approach described in Figure 5 was validated by the growth assay and Western blotting analysis.  $\Delta cyc3$  did not grow on non-fermentable carbon source, because CCHL encoded by *cyc3* is required for cell growth on non-fermentable carbon source. The growth defect was recovered by expressing CYC3, CYC3HA and pCytb<sub>2</sub>\_CYCHA respectively, whereas an empty vector or a vector encoding pCytb<sub>2</sub>(A63P)\_CYC3HA failed to rescue the growth defect (Figure 7A, B). Moreover, the mature protein size of expressed pCytb<sub>2</sub>-CCHL or pCytb<sub>2</sub>(A63P)-CCHL in  $\Delta cyc3$  were monitored by Western blotting with an  $\alpha$ -HA antibody. pCytb<sub>2</sub>-CCHL showed additional bands of smaller size compared to pCytb<sub>2</sub>(A63P)-CCHL, as pCytb<sub>2</sub>-CCHL is inserted and cleaved by Imp. Thus it supports that pCytb<sub>2</sub>(A63P)-CCHL cannot be inserted into the membrane but translocated to the matrix (Figure 7C). As

pCytb<sub>2</sub>\_CYC3HA rescues the growth defect of  $\Delta cyc3$  on non-fermentable carbon media when it is sorted to the IM by the TIM23 complex, the  $\Delta cyc3$  transformants expressing pCytb<sub>2</sub>(A63P)\_CYC3HA, in theory, would grow on non-fermentable carbon source if a mutant Tim23 or Tim17 sorts the fusion protein to be inserted into the IM.



**Figure 7. Validation of TIM23 random mutagenesis screening.**

(A) Yeast strain ( $\Delta cyc3$ ) was transformed with an empty vector or a vector encoding CYC3, CYC3HA, pCytb<sub>2</sub>\_CYC3HA or pCytb<sub>2</sub>(A63P)\_CYC3HA. The transformants were streaked on YPG (non-fermentable carbon source) plate and incubated at 30°C for two days.  $\Delta cyc3$  did not grow on YPG. The growth defect was rescued by expression of CYC3, CYC3HA and pCytb<sub>2</sub>\_CYC3HA. Expression of pCytb<sub>2</sub>(A63P)\_CYC3HA did not restore the growth defect.

(B) The table shows the summary of the growth assay.

(C)  $\Delta cyc3$  transformed with pCyb<sub>2</sub>\_CYC3HA or pCyb<sub>2</sub>(A63P)\_CYC3HA was grown in fermentable carbon source at 30°C overnight. Proteins were prepared through crude membrane fractionation and were subjected to SDS-PAGE and Western blotting. Bands were detected with  $\alpha$ -HA antibody.

## ***Tim17 mutants capable of inserting the fusion protein to the IM are selected***

To investigate the regions of the TIM23 complex involved in lateral insertion of mitochondrial IM proteins containing proline residue, random mutagenesis study was done. As Tim23 and Tim17 are central components of the main channel for protein substrates, Tim23 and Tim17 were randomly mutagenized for screening. Random mutagenesis was done by using 'Diversify PCR Random mutagenesis kit' from 'Takara', for stable error rates and PCR efficiency. A large number of randomly mutated Tim23 and Tim17 were then combined into p426GPD vector by Gibson assembly method which aids in vitro homologous recombination. They were overexpressed under GPD promoter. Ligated vectors expressing various mutant Tim23 or Tim17, were co-transformed with pCytb<sub>2</sub>(A63P)-CCHL into  $\Delta cyc3$  strain.  $\Delta cyc3$  needs CCHL in the IMS for cell growth on non-fermentable carbon source, so only  $\Delta cyc3$  cells having mutant Tim23 or Tim17 which allows insertion of pCytb<sub>2</sub>(A63P)-CCHL would grow on non-fermentable carbon source.

After co-transformants were grown on –Leu-Ura (glucose) plate, they were then transferred to non-fermentable carbon source plate for the selection. Some of the transformants grew on non-fermentable carbon source plate, and they were streaked on a new plate again. Survived colonies were referred to as probable candidates with enhanced membrane insertion of the fusion protein. To monitor the processing of the fusion protein, Western blotting analysis was done. Following the theory of the selection scheme, viable cells have inserted pCytb<sub>2</sub>(A63P)-CCHL forms so that CCHL in the IMS rescues the growth defect. In this case, the fusion protein should be detected as smaller size of bands as in the left lane of Figure 7C. However, among the selected positive colonies on non-fermentable carbon source, only three colonies having Tim17 mutants showed smaller size of pCytb<sub>2</sub>(A63P)-

CCHL (Figure 8A, B). So far, no Tim23 mutants yet selected, although it is well known that it forms the pore of the channel.

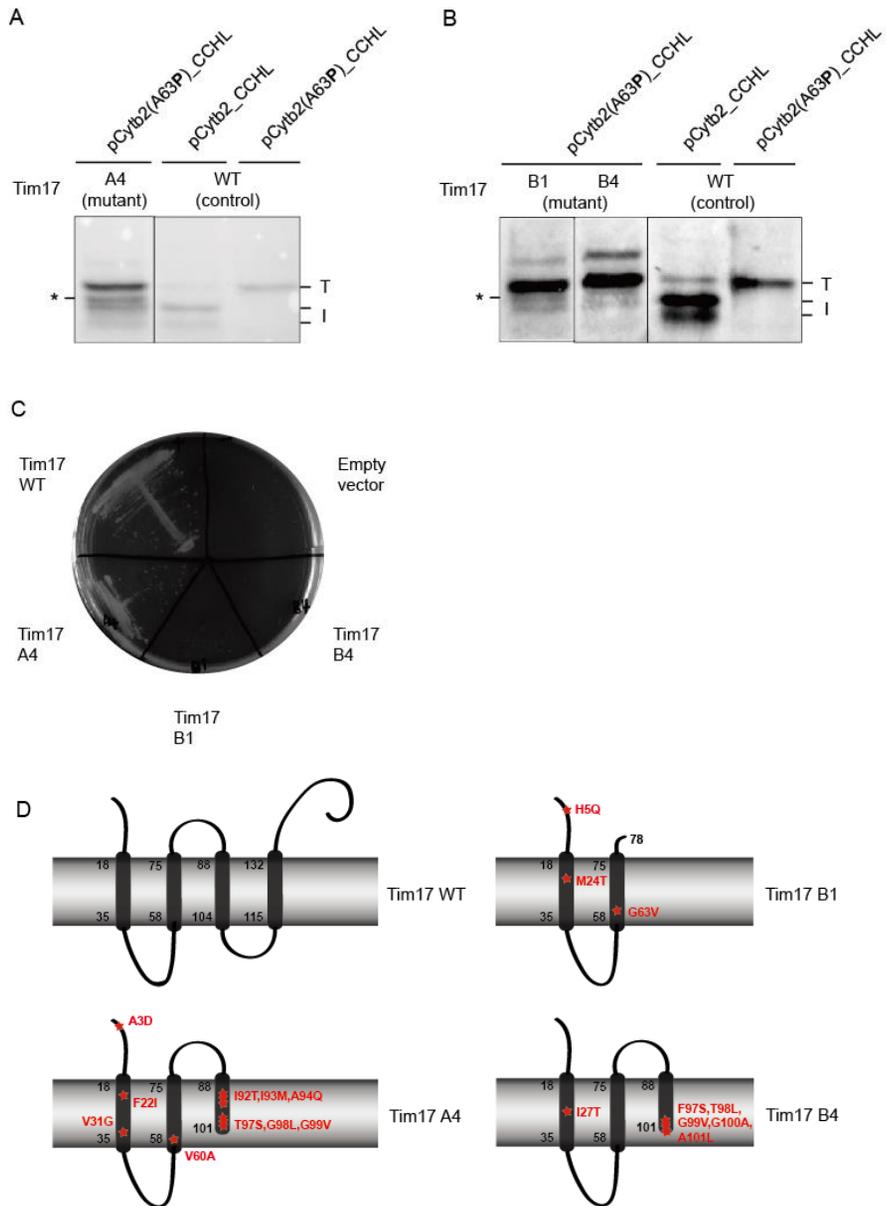
Three Tim17 mutants showing enhanced insertion activity for the model fusion protein containing proline residue, are isolated and identified (Figure 8C). Tim17 mutant indicated as Tim17 A4, has ten amino acid changes especially in the 3<sup>rd</sup> transmembrane domain (TMD) and is truncated at residue 101. Tim17 B1 has three amino acids changes and is truncated at residue 78. Tim17 B4 has six amino acid changes and is also truncated at residue 101 like Tim17 A4. Exchanged residues in these Tim17 mutants could be significant for regulating lateral insertion of proteins into the IM, but further studies are needed to investigate the effects of each mutated residue.

To investigate whether Tim17 mutants are functional without wild type Tim17, growth test was conducted. Each mutant Tim17 in p424GPD vector was introduced into a Tim17 shuffling strain,  $\Delta tim17$  strain with a wild type copy of the protein encoded on a Ura Plasmid, p416. The ability of the mutant Tim17 to support the growth was then assessed on a medium containing 5-fluoroorotic acid (5-FOA) that selects against Ura plasmid. Among three mutants, only Tim17 A4 was viable on 5-FOA plate. Wild type Tim17 in p314 vector and p424GPD empty vector were used for positive and negative control, respectively (Figure 8D).

Interestingly, Tim17 A4 and B4 are truncated at the same position, lacking the loop in matrix side. A recent study reported that the residue 105 in the 3<sup>rd</sup> loop of Tim17 is involved in binding with Tim44 [37]. Tim44 recruits the PAM motor to the TIM23 complex, and lack of Tim44 recruitment to the TIM23 is shown causing dissociation of Pam18-Pam16 pair from Tim23-Tim17 core. Tim17 mutants lack the residue 105 in the loop, therefore its recruitment of Tim44 may be impaired so the interaction with the PAM motor might be impaired. Because the PAM motor is crucial for complete translocation of mitochondrial proteins into the matrix, it is reasonable to

assume that pCytb<sub>2</sub>(A63P)-CCHL is inserted into the membrane rather than translocated to the matrix when Tim17 mutant is expressed.

As it is suggested that impaired interaction with the PAM motor can insert pCytb<sub>2</sub>(A63P)-CCHL into the mitochondrial IM, further test was done to identify the relationship between the PAM motor and lateral insertion via the TIM23 complex, by Western blotting analysis (Figure 8A). In *pam16-3* which is a mutant strain having defective import motor, pCytb<sub>2</sub>(A63P)-CCHL was inserted to the IM (Figure 9A). These data suggest that the transmembrane domain carrying proline residue can be laterally inserted into the mitochondrial IM when the import motor is defective. In addition, membrane insertion efficiency of pCytb<sub>2</sub>(A63P)-CCHL in the absence of Mgr2 was tested. Mgr2 is a subunit of TIM23 complex, and is shown to be involved in lateral insertion of proteins into the IM; the absence of Mgr2 increases membrane protein insertion whereas overexpression decreases. In the absence of Mgr2, pCytb<sub>2</sub>(A63P)-CCHL was inserted to the IM (Figure 9B), and the size of the processed protein was similar with that observed in Tim17 mutants and in *pam16-3*.

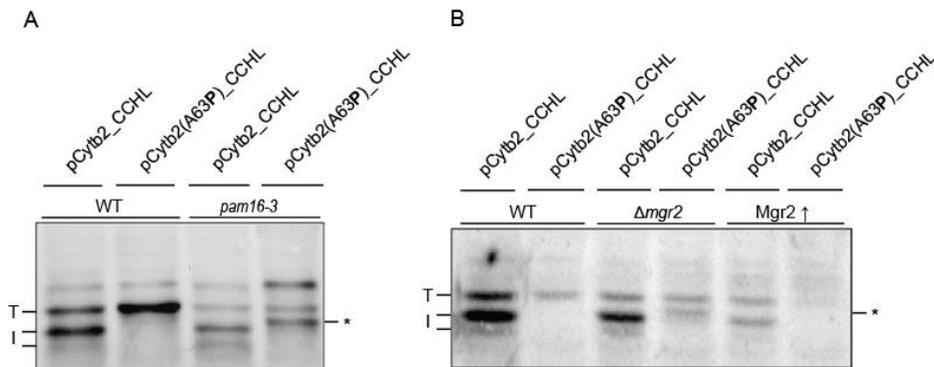


**Figure 8. Selection and analysis of Tim17 mutants that rescue the growth defect of  $\Delta cyc3$  on non-fermentable carbon media by random mutagenesis**

(A) (B) Mutant Tim23/Tim17 libraries were transformed into  $\Delta cyc3$  with pCytb<sub>2</sub>(A63P)-CCHL and incubated at 30°C for 2 days. Transformants in – Leu-Ura were transferred to non-fermentable carbon source YPG plate for the selection, and survived colonies were once more streaked to another YPG plate. Viable cells were then subjected to crude membrane fractionation for protein preparation, SDS-PAGE, and Western blotting. For control, pCytb<sub>2</sub>-CCHL and pCytb<sub>2</sub>(A63P)-CCHL were respectively transformed into  $\Delta cyc3$  and each transformant was examined the same way. Tim17 mutants A4, B1, and B4 are shown with the control. T indicates a translocated form of the fusion protein and I is an inserted form. The additionally detected band between T and I is indicated as asterisk (\*).

(C) From transformants in (A) and (B), Tim17 mutant A4, B1, and B4 were isolated and cloned into a p424GPD vector. Tim17 mutants in p424GPD vector were then transformed into a  $\Delta tim17$  strain containing wild type Tim17 in p416 vector. Transformants on –His-Trp were streaked on –His-Trp plate containing 5-fluoroorotic acid (5-FOA) for the selection against Ura plasmid, for removing the wild type Tim17. For control, wild type Tim17 in a p314 vector and an empty p424GPD vector were transformed into  $\Delta tim17$  plus p416 Tim17 and investigated likewise. After kicking out p416 Tim17 on 5-FOA plate, wild type Tim17 (positive control) and mutant Tim17 A4 were viable.

(D) Schematic diagram of wild type Tim17 and mutants is shown.



**Figure 9. Defective import motor and deletion of Mgr2 affects insertion of pCytb<sub>2</sub>(A63P)-CCHL**

(A) (B) pCytb<sub>2</sub>-CCHL and pCytb<sub>2</sub>(A63P)-CCHL were transformed into *pam16-3* mutant strain and isogenic wild type strain, and into Mgr2 deleted ( $\Delta mgr2$ ), Mgr2 overexpressed (Mgr2 $\uparrow$ ) and isogenic wild type strain. All transformants were analyzed by crude membrane fractionation, SDS-PAGE, and Western blotting as described in Figure 8. In wild type strain, pCytb<sub>2</sub>-CCHL was inserted into the membrane (indicated as I) and pCytb<sub>2</sub>(A63P)-CCHL was translocated into the matrix (T). In *pam16-3* and  $\Delta mgr2$  strains, pCytb<sub>2</sub>(A63P)-CCHL showed the band between T and I, indicated as \* which is assumed to be inserted into the IM.

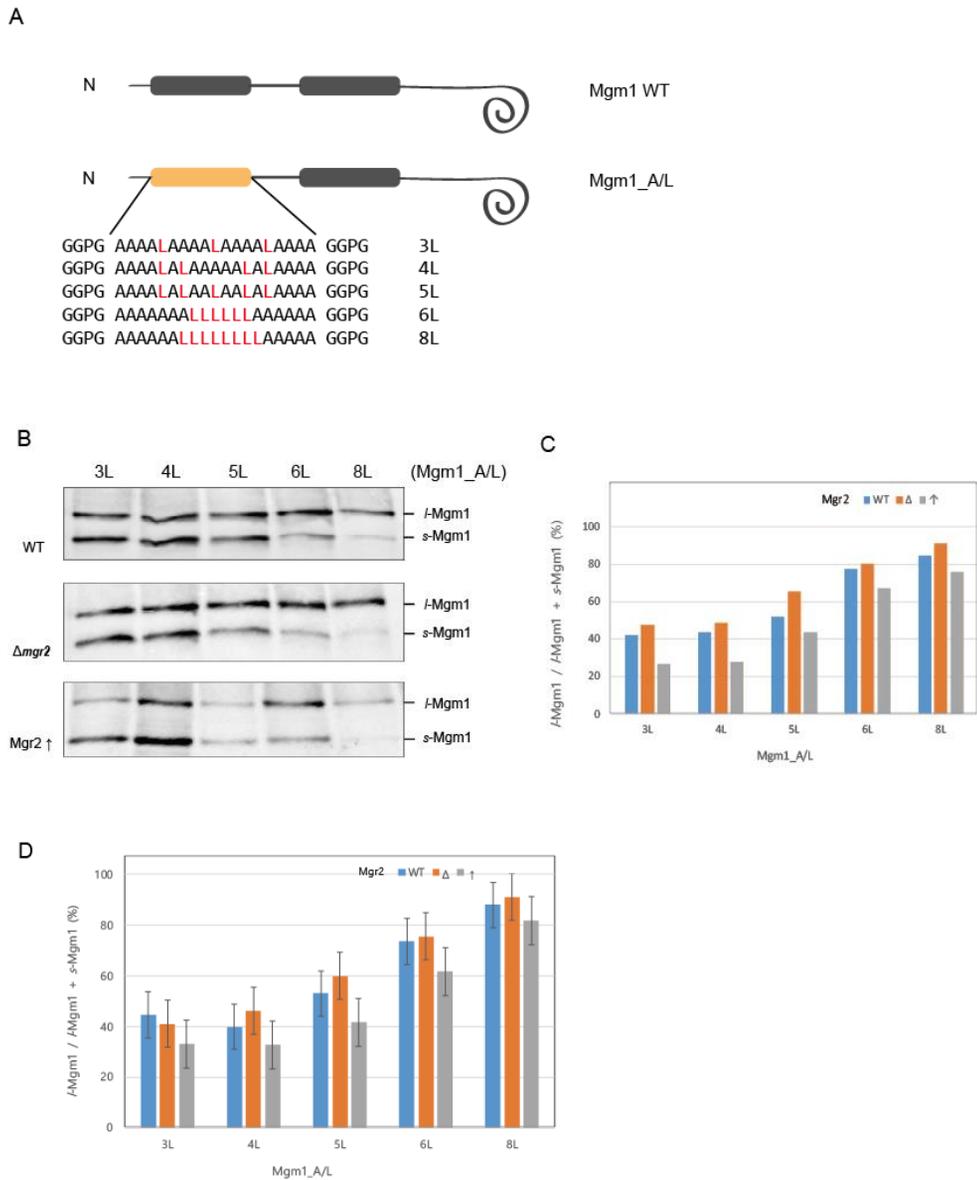
## ***Mgr2 is involved in setting the hydrophobicity threshold required for mitochondrial IM membrane protein insertion***

While overall hydrophobicity required for transmembrane domain (TMD) insertion into the mitochondrial IM is shown comparable to that is required in the endoplasmic reticulum (ER) membrane, differences in threshold hydrophobicity and effects of flanking charged residues have been observed. But what contributes these differences and the components that define the hydrophobicity required for the TMD insertion into the IM remains elusive.

Mgr2 was recently identified and reported to act as a lateral gatekeeper based on the observation that the absence of Mgr2 facilitates membrane protein insertion of Mgm1 and Cytb<sub>2</sub> while overexpression delays. So, it has raised a question of whether Mgr2 is involved in defining hydrophobicity requirement for the TMD insertion into the IM. To investigate this, Mgr2 deletion (*mgr2*Δ), Mgr2 overexpression (Mgr2↑) and the corresponding wild type strain were obtained from Dr. Pfanner [22,31].

In this study, Mgm1 was used as a model protein. It naturally generates two forms, *l*-form and *s*-form, depending on the sorting of the 1<sup>st</sup> TMD and each form indicates membrane inserted and translocated form, respectively. To test whether Mgr2 regulates the hydrophobicity required for membrane protein insertion into the IM, Mgm1\_A/L variants whose 1<sup>st</sup> TMD is replaced by 19 alanine(*n*)/leucine(*l9-n*) stretch were expressed in Mgr2 deleted, Mgr2 overexpressed, and the corresponding wild type strains. Tested Mgm1\_A/Ls are Mgm1\_3L, 4L, 5L, 6L, and 8L, with increasing hydrophobicity from 3L to 8L (Figure 9A). The relative amounts of *l*-Mgm1 which indicates membrane insertion were increased with higher number of leucines in all of Mgr2 strains (Figure 9B, C). Overall, when compared to WT, the relative amounts of *l*-Mgm1 were increased in the absence of Mgr2, while those were decreased upon the overexpression of Mgr2. To examine in more detail, four independent experiments (three for Mgm1\_3L) was done and the

average of all data is shown in Figure 9D. While 50% membrane insertion reached for  $n \doteq 5$  in WT and when Mgr2 was deleted, increased to  $n \doteq 5-6$  upon overexpression of Mgr2. Thus, these data suggest that Mgr2 has a role in regulating the hydrophobicity requirement for the membrane protein insertion into the mitochondrial IM.



**Figure 10. Mgr2 has an effect on the membrane insertion of Mgm1 carrying varying hydrophobicity**

(A) Schematics of wild type Mgm1 and Mgm1\_A/Ls. The 1<sup>st</sup> TMD of Mgm1 WT was exchanged to alanine/leucine residues. Sequences of Mgm1\_A/L 1<sup>st</sup> TMD are shown.

(B) Mgm1\_A/Ls with indicated number of leucines were expressed in WT,  $\Delta mgr2$ , and Mgr2 $\uparrow$ . Proteins were TCA precipitated from overnight cultures in glucose medium. Proteins were then subjected to SDS-PAGE and Western blotting to distinguish *l*-Mgm1 and *s*-Mgm1. The protein bands were detected using  $\alpha$ -HA antibody.

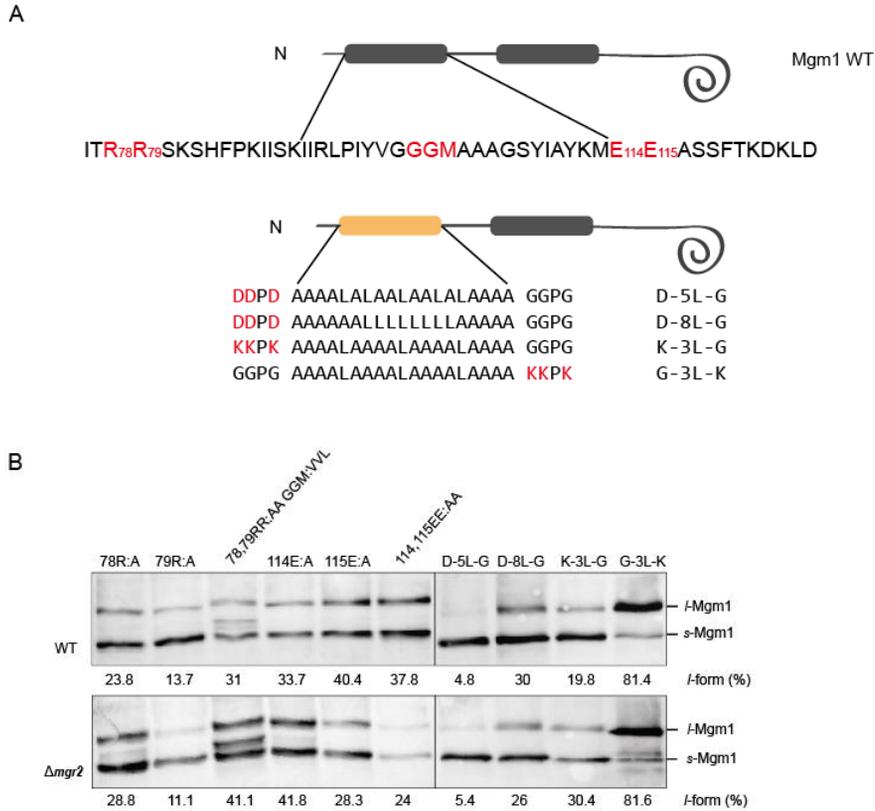
(C) Relative amounts of *l*-Mgm1 ( $l\text{-Mgm1} / l\text{-Mgm1} + s\text{-Mgm1}\%$ ) was quantified.

(D) Relative amounts of *l*-Mgm1 were calculated. Means  $\pm$ S.E. from four independent experiments are shown (error bars).

### ***Mgr2 senses the charges preceding the TMD of Mgm1***

In recent study, Mgr2 was reported to block the insertion of Cytb2 with mutated N-terminal flanking charges. The sorting of the 1<sup>st</sup> TMD of Mgm1 is dependent on the flanking charged residues [38]. These previous data raised a question whether Mgr2 recognizes the charged residues flanking TMD of Mgm1, and regulates the insertion.

To test this hypothesis, the sorting of a set of Mgm1 mutants were monitored in Mgr2 deleted, and corresponding wild type strains. These mutants have replaced charged residues flanking the 1<sup>st</sup> TMD of Mgm1 to alanine residues, such as positively charged R78 R79, and negatively charged E114 and E115 (Figure 11A). In addition, for other mutants, positively charged lysine (K) or negatively charged aspartic acid (D) was added in the upstream or the downstream of the 1<sup>st</sup> TMD of Mgm1\_A/L (Figure 11A). When the upstream flanking charged residue was mutated, the sorting of mutant proteins was affected by the deletion of Mgr2. The sorting of Mgm1\_R78A was mildly affected by the deletion of Mgr2 and the sorting of Mgm1\_R78A R79A GGM:VVL was also Mgr2 dependent (Figure 11B). Mgm1\_K-3L-G mutant which have three K in the upstream of the TMD was shown to be more affected by the absence of Mgr2. However, the mutations of the downstream flanking charged residues did not change the Mgm1 sorting dependency on Mgr2 with the exception of Mgm1\_E114A. These data suggest that the charged residues preceding the 1<sup>st</sup> TMD of Mgm1 is critical for Mgr2-regulated insertion of membrane protein into the mitochondrial IM. For further analysis, analysis with Mgr2 overexpression strain and replication of independent experiments are in need.



**Figure 11. Mgr2 senses the upstream flanking charged residues of Mgm1**

(A) Sequence of the Mgm1 (from residue 76 to 125) and Mgm1 mutants is presented.

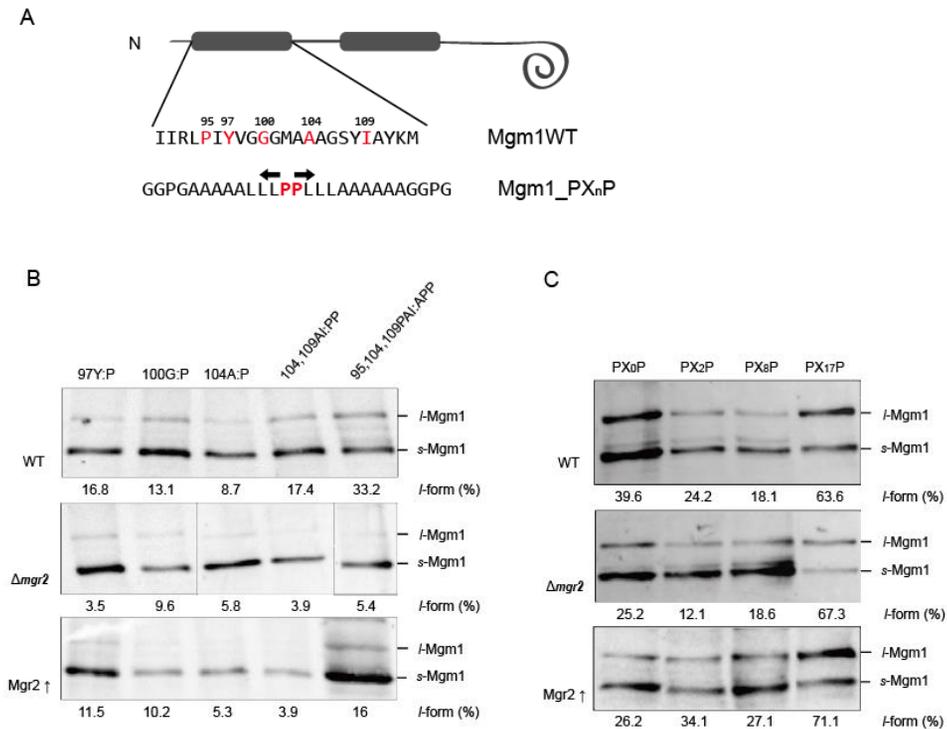
(B) Flanking charged mutants of Mgm1 were expressed in WT,  $\Delta mgr2$ . Proteins were prepared and separated as described in Figure 10. Relative amounts *l*-Mgm1 (%) were quantified and indicated below the blot.

## ***Deletion of Mgr2 shows an effect on sorting of Mgm1 carrying proline mutations***

Mgm1 variants with various hydrophobicity or charged residues flanking the TMD were partially affected by the level of Mgr2. Lastly, whether Mgr2 senses the proline residue in a TMD and regulates the protein insertion was tested. A proline residue is known to act as a  $\alpha$ -helical structure breaker. There is a tendency to have no or less proline residue in a TMD of mitochondrial membrane protein sorted by the stop transfer pathway, while the ER membrane is quite tolerable to proline containing TMD. Besides, for mitochondrial IM proteins, introduction of proline residue into the TMD can make the membrane protein mistargeted to the matrix as pCytb<sub>2</sub>(A63P)-CCHL.

To test the role of Mgr2 in dealing with proteins containing proline in a TMD, some amino acids in the 1<sup>st</sup> TMD of Mgm1 were mutated to proline residues (Figure 12A), and those Mgm1 mutants were expressed in Mgr2 deleted, Mgr2 overexpressed, and corresponding wild type strains. Mgm1 variants which contains the alanine and leucine stretch with two prolines introduced from the middle to each ends of the 1<sup>st</sup> TMD were also investigated (Figure 12A). When one or two amino acids in the 1<sup>st</sup> TMD of Mgm1 were mutated to proline residues, the relative amounts of *l*-Mgm1 were quite decreased when Mgr2 was overexpressed, and in the absence of Mgr2 (Figure 12B). When two prolines were introduced in the 1<sup>st</sup> TMD of Mgm1\_A/L, which indicated as Mgm1\_PX0P, showed similar tendency when Mgr2 was deleted. The relative amounts of *l*-Mgm1\_mutants were decreased in the absence of Mgr2 whereas increased upon overexpression of Mgr2, except for Mgm1\_PX0P (Figure 12C). These results show that the TMD carrying proline residue can be more inserted into the mitochondrial IM in the absence of Mgr2. Thus, it suggests that Mgr2 is involved in lateral insertion of proline containing proteins into the mitochondrial IM. Further studies are in demand

to investigate more detailed role and mechanism of Mgr2.



**Figure 12. Mgr2 has an impact on sorting of Mgm1 with proline mutation in TMD**

(A) Sequence of the 1<sup>st</sup> TMD of Mgm1 and schematics of Mgm1\_PX<sub>n</sub>P is shown. n indicates the number of residues between two prolines residues.

(B) Mgm1 mutants carrying proline were expressed in WT,  $\Delta mgr2$  and Mgr2 $\uparrow$ . Proteins were prepared and separated as described in Figure 10. Relative amounts *l*-Mgm1 (%) were quantified and indicated below the blot.

(C) Mgm1\_PX<sub>n</sub>Ps described in (A) were tested as in (B).

## DISCUSSION

Mitochondria are involved in many cellular processes, thus mitochondrial dysfunction is harmful to the cell, resulting in various diseases. Proper localization of mitochondrial proteins is essential for functional mitochondria, so understanding of how mitochondrial proteins are sorted into the right subcompartments is important.

For mitochondrial inner membrane (IM) proteins, the TIM23 complex is the main channel for N-terminal presequence containing proteins. Some membrane proteins are laterally inserted into the IM directly by the TIM23 complex (strop-transfer pathway), whereas other IM proteins are translocated into the matrix through TIM23 complex. They are subsequently inserted into the IM by the OXA1 complex from the matrix (conservative sorting pathway).

The majority of integral membrane proteins have tightly packed transmembrane (TM)  $\alpha$ -helices, and the formation of an  $\alpha$ -helical structure and overall hydrophobicity are crucial determinants for translocase-mediated recognition and insertion of a TM domain (TMD) into the lipid bilayer. While overall hydrophobicity required for a TMD insertion into the mitochondrial IM is shown comparable to that required in the endoplasmic reticulum (ER) membrane, differences in threshold hydrophobicity and effects of flanking charged residues have been observed. In addition, Proline residue in a TMD is another strong determinant of membrane insertion. The impact of proline residues on the insertion of hydrophobic segment is shown in both the mitochondrial IM and ER membrane, but the mitochondrial IM is less tolerable to proline than the ER membrane. In mitochondria, there is a

tendency of lacking proline residue in the TMDs of laterally inserted proteins, and introduction of proline residue to a TMD can make mitochondrial IM proteins to be mistargeted to the matrix. However, what contributes these differences is currently unknown.

To identify the regions or residues in the TIM23 components critical for lateral insertion of proline containing proteins into the IM, the random mutagenesis was done for genetic screening. The central channel forming subunits, Tim23 and Tim17 were randomly mutagenized and mutants of them with enhanced membrane insertion activity were selected. In this approach, Tim17 mutant with enhanced membrane insertion of a TMD containing proline residue that normally translocates to the matrix is identified. This Tim17 mutant is truncated at the C-terminus, lacking the loop region in the matrix. A recent study reveals that this loop region in the matrix is crucial for interaction with an import motor complex in the matrix. Therefore, the TIM23 complex with Tim17 mutant lacking this loop region may have impaired interaction with the motor complex. As the import motor is required for complete translocation of proteins to the matrix, Tim17 mutant might have caused decreased translocation activity, so the model protein with proline was inserted into the IM in Tim17 mutant. Another study shows that the same model protein containing proline residue can be inserted in a mutant yeast strain having defective import motor.

In second approach, I focused on Mgr2 which is recently identified subunit of the TIM23 complex. It is known to be involved in interaction of the TIM23 core complex and in lateral insertion of mitochondrial IM proteins. A recent study shows that membrane insertion efficiency of proteins is increased in the absence of Mgr2, and decreased upon overexpression of Mgr2. Since what defines the hydrophobicity threshold in mitochondrial IM is unknown, I investigated whether Mgr2 has a role in setting hydrophobicity threshold and regulating lateral insertion of membrane proteins. The result

shows that the hydrophobicity threshold is decreased in the absence of Mgr2, whereas increased upon the overexpression of Mgr2. In addition, when Mgr2 is absent, membrane insertion efficiency of the TMD having alterations in flanking charged residues is increased, while that of the TMD containing mutated proline residues is decreased. In sum, these data suggest that Mgr2 is involved in lateral insertion of mitochondrial IM proteins, through setting the hydrophobicity threshold and sensing determining features of membrane proteins.

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## 국문초록

*Saccharomyces cerevisiae* 에서 절단 가능한 전서열을 가진 미토콘드리아 내막 단백질은 핵에 암호화되어 있고, 내·외막 트란스로카제 (TOM•TIM23 복합체)에 의해 내막으로 삽입된다. TIM23 복합체는 또한 단백질을 미토콘드리아 기질로 수송하는 역할도 담당한다. 그러나 TIM23 복합체가 어떻게 전구체 단백질의 최종 위치 신호를 인식하여 단백질을 내막 또는 기질로 보내는지에 대해서 많이 알려져있지 않다. TIM23 복합체에 의한 미토콘드리아 단백질 삽입 기작을 더 심도깊게 이해하기 위해, 두가지 방법으로 연구를 진행하였다. 첫 번째로, TIM23 복합체에서 내막 단백질의 삽입에 중요한 부위를 알아내기위해 유전자 스크리닝을 수행하였다. 수행 결과, 막관통부위에 Proline을 가져 기질로 수송되는 모델 단백질의 막 삽입 효율이 높아진 Tim17의 돌연변이를 발견하였다. 이 돌연변이의 서열을 분석해본 결과, 카르복시 말단에서 잘려 기질에 있는 고리 부분이 없는 Tim17의 돌연변이로 밝혀졌다. 이 고리 부분은 기질에서 단백질의 수송을 담당하는 수송 모터와의 상호작용에 중요하다고 알려져있다. 따라서 Tim17의 돌연변이로 인해 TIM23 복합체와 수송 모터와의 결합이 약해진 것이, 모델 단백질의 수송효율이 떨어지고 내막으로 삽입된 이유일 것으로 추측된다.

두 번째 연구는 최근에 새로운 TIM23 복합체의 구성요소 밝혀진 Mgr2의 기능을 밝히는데 집중하였다. Mgr2가 없을 때 TIM23 복합체의 중심과 Tim21, 호흡연쇄복합체와의 상호작용이 저해되며, Mgr2가 TOM-TIM23 복합체의 결합에도 역할을 한다는 것이

알려졌다. 또한, 다른 연구 결과에서는 Mgr2가 없을 때 막 단백질의 삼입 효율이 증가하고, Mgr2가 과발현될 때 막 단백질의 삼입이 지연되는 것으로 나타났다. 따라서 Mgr2가 막 단백질의 삼입을 조절하는 역할을 하는지 알아보기 위해, Mgr2 돌연변이에서 다양한 특징의 서열을 가진 Mgm1 변형 단백질들의 막 삼입 효율이 어떻게 달라지는지 관찰하였다. 연구 결과, Mgr2가 과발현되면 막관통도메인이 막에 삼입되는 데에 필요한 소수성한계점이 증가하였고, Mgr2가 제거되었을 때에는 감소하였다. 막관통도메인 근처의 전하를 띠는 아미노산에 돌연변이를 가지는 Mgm1 변형 단백질에 대해서는, Mgr2가 없을 때 단백질의 삼입이 증가하는 양상을 보였다. 또한, Mgr2가 없을 때 proline을 가지는 막관통도메인의 삼입은 감소하였다. 이러한 연구 결과로 보아, Mgr2가 막관통도메인의 주요 특징을 감지하고 막 단백질의 삼입을 조절하는 역할을 하는 것으로 보여진다.

주요어: TIM23 복합체, 막 단백질 삼입, Tim17, Mgr2, 막관통도메인, Mgm1