이학석사학위논문

미토콘드리아 내막 단백질 형성에서 TIM23 complex와 m-AAA protease의 역할

Recognition and Sorting of Mitochondrial Inner Membrane Proteins by the TIM23 Complex and the m-AAA Protease

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Recognition and Sorting of Mitochondrial Inner Membrane Proteins by the TIM23 Complex and the m-AAA Protease

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ABSTRACT

The TIM23 complex mediates translocation of proteins into two subcompartments of mitochondria; the inner membrane (IM) and the matrix, but how this complex is arranged in the IM and how it distinguishes targeted location of incoming polypeptides and opens the pore either transversally or laterally still remain elusive. Through site-directed mutagenesis of specific residues within the channel forming subunits, Tim17p and Tim23p, and translocation assay with various substrates of the TIM23 translocon, we attempted to elucidate their function in protein sorting. The mutagenesis study demonstrates that the second transmembrane domain (TMD2) of Tim23p plays a pivotal role. Particularly, mutation at the matrix side of the TMD2 affects the membrane insertion of proteins.

The m-AAA protease participates in a quality control system of mitochondria and processing of specific proteins. However, it is yet to be revealed how it recognizes proteins for degradation and processing. To survey the characteristics of proteins that the m-AAA complex senses and subsequently exerts its ATPase activity, we utilized a set of Mgm1p variants with diverse sequence contexts. Our results show that depending on where the proline or charged residues are positioned within the TMD, the m-AAA protease differentially recognizes and dislocates the segments.

Key words: the TIM23 complex, mitochondrial protein sorting, memebrane insertion, Tim23p, Tim17p, the m-AAA protease, mitochondrial protein quality control

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INTRODUCTION

Except a few subunits of the respiratory chain complexes and the F_1F_0-ATP synthase, most mitochondrial proteins are encoded by the nuclear DNA. These nuclear-encoded mitochondrial proteins are synthesized as a precursor in the cytosol in close proximity to the mitochondria [1][2] and targeted and sorted to the right mitochondrial subcompartments. Mitochondria are composed of two membranes, the outer membrane (OM) and the inner membrane (IM), and two aqueous compartments, the intermembrane space (IMS) and the matrix. Translocation of mitochondrial precursor proteins to its functional location is facilitated by various machineries [3].

Mitochondrial protein sorting and machineries

Most mitochondrial precursors pass through the common entry gate, the translocase of the outer membrane (TOM) complex and proceed with their translocation to the designated subcompartment following different pathways [3]. Distinct downstream import pathways are facilitated by diverse translocases such as the SAM, Mim1, MIA, OXA, TIM22 and TIM23 complexes. Insertion of β-barrel proteins and some of α-helical proteins to the mitochondrial OM is mediated by the sorting and assembly machinery (SAM) complex, whereas the outer membrane insertion of α-helical proteins with a transmembrane domain (TMD) at the N-terminus engages mitochondrial import 1 (Mim1) [4]. Oxidative folding and import of IMS proteins with Cys motifs are aided by the mitochondrial intermembrane space assembly (MIA) complex [5].

There are three complexes known to be involved in the generation of IM proteins. The oxidase assembly 1 (OXA1) complex drives protein insertion to the IM from the matrix side and both translocases of the inner membrane (TIM) 23 and 22 sort proteins entering from the IMS side. The TIM22 complex is mainly involved in sorting of multi-spanning proteins, like carrier proteins, and the TIM23 complex directs proteins with an amphipathic α-helical presequence to the IM or the matrix [4].

The TIM23 complex

The TIM23 complex mediates transport of presequence carrying mitochondrial

proteins across or into the mitochondrial inner membrane (IM). This complex interacts with preproteins and the complex formation has been shown to be dynamically regulated depending on the destination of the translocating substrate. If preproteins are integrated into the membrane, the core subunits of this complex (Tim23p, Tim17p and Tim50p) associate with Tim21p and the respiratory chain complex III and IV, whereas if proteins are imported into the matrix, the presequence translocase-associated import motor (PAM) is recruited to the TIM23 complex [6].

Electrophysiological studies suggest that the TIM23 complex is voltage sensitive and has a twin pore structure [7, 8]. Fluorescence mapping and cross-linking studies suggested the arrangement of subunits and structural changes of the TIM23 complex during substrate import [9, 10]. Yet, challenges in reconstitution of membrane embedded protein complex and crystallography impede us from getting high-resolution structure of the TIM23 complex. Among subunits of this complex, Tim23p and Tim17p form the protein-conducting channel [7]. These subunits are homologous and both contain four TMDs with the C-and N-terminal ends facing the IMS (Fig. 1). However, little is known how they are organized in the IM with other subunits of the TIM23 complex. Further studies on how they work together to recognize, distinguish the sorting pathways, and finally translocate the substrates are in demand.

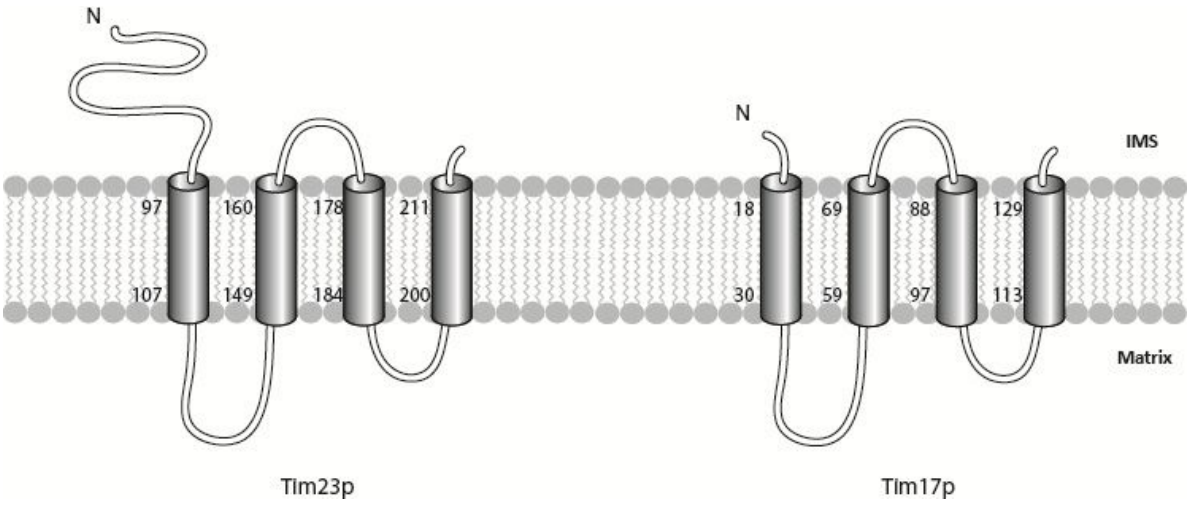


Fig. 1 Topology of Tim23p and Tim17p Tim23p and Tim17p are homologous with the same topology, both N-and C- terminal ends facing the IMS. In this study, mutations were introduced at the ends of each TMD either individually or in combination.

Stop-transfer and conservative sorting pathways

Most presequence carrying mitochondrial IM proteins are partitioned to the membrane using two alternative sorting pathways, stop-transfer and conservative sorting pathways [11, 12]. Proteins, partitioned by the stop-transfer pathway, are arrested at the TIM23 translocon and laterally released to the membrane. Whereas proteins following the conservative sorting pathway, are fully translocated to the matrix and inserted to the IM with the help of the OXA1 complex, in the same manner as the IM proteins encoded by the mitochondrial DNA. It is suggested that proline residues in TMDs and hydrophobicity of TMD may be indicators that the TIM23 complex distinguish for sorting of the substrates [13].

Alternative topogenesis of Mgm1p

Mgm1p, a dynamin-like GTPase, is a substrate of the TIM23 complex. This protein has two isoforms, a membrane anchored long form (*l*-Mgm1p) and a soluble short form (*s*-Mgm1p) that reflects either insertion or import of the first TMD of this protein into or across the IM, respectively. At steady state level, roughly forty percent of the first TMD of Mgm1p is recognized by the TIM23 complex and laterally released to the IM, generating long form (*l*-Mgm1p). The remaining molecules slip through the pore, and the second TMD of Mgm1p is processed by the rhomboid like protease, Pcp1p, releasing the short form (*s*-Mgm1p) to the IMS [20] (Fig. 2). This feature of Mgm1p allows us to access the efficiency of protein translocation vs. membrane insertion by various *tim17* and *tim23* mutants.

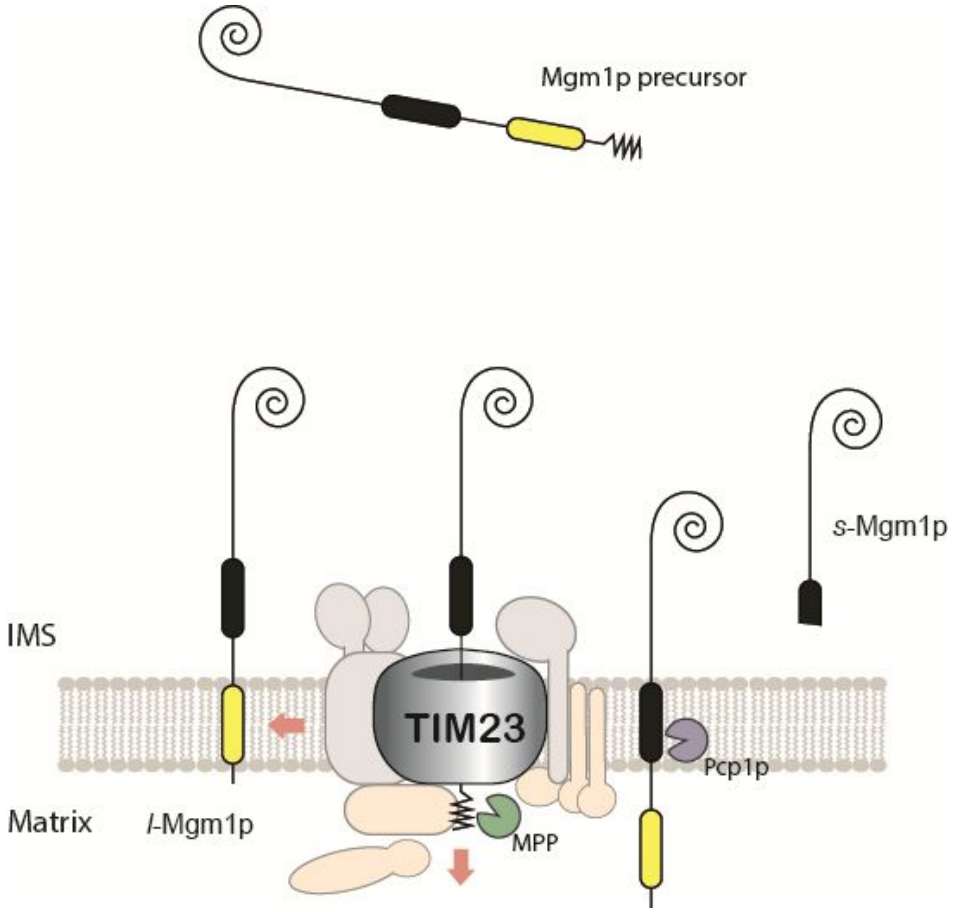


Fig. 2 Alternative topogenesis of Mgm1p Mgm1p is a protein sorted by the TIM23 complex. When this protein arrives at the TIM23 translocon, the presequence is cleaved by the matrix processing peptidase (MPP). If the first TMD (yellow) of Mgm1p is recognized by the TIM23 translocon as membrane insertion signal, it is laterally released to the inner membrane, generating *l*-Mgm1p (long form of Mgm1p). When the first TMD of Mgm1p imported to the matrix, the second TMD of this protein is processed by the inner membrane protease, Pcp1p, yielding *s*-Mgm1p (short form of Mgm1p)

The m-AAA protease

Because of the spatial limitation of the translocons, folding and complex formation of mitochondrial proteins need to occur during or after translocation. While this happens, various stress conditions can lead to misfolding of proteins. Also, during complex formation, imbalanced level of subunits can generate orphan subunits. These misfolded and non-assembled proteins should be removed and the m-AAA (ATPase associated with diverse cellular activities) protease has been implicated as an essential player in this process. [14].

The m-AAA protease is a hetero-oligomeric complex containing Yta10p and Yta12p. These subunits have 2 TMDs at the N-terminus, large AAA+ domain and a metal-dependent proteolytic domain facing the matrix side [14]. So far, various cellular functions of the m-AAA proteases have been observed. Firstly, this complex recognizes unfolded proteins in the IM and mediates their degradation [15], but direct evidence that the m-AAA protease actually exerts proteolytic activity still awaits. Secondly, it is involved in processing and maturation of MrpL34p, a subunit of mitochondrial ribosome, and cytochrome c peroxidase (Ccp1p) [16, 17]. This additional activity of the m-AAA protease draws attention to how this complex discriminates one that should be completely degraded from the other that need to be processed. It is suggested that the folding status of proteins may act as an indicator for processing by halting further degradation [18]. Lastly, it has been shown that the m-AAA protease dislocates membrane-embedded proteins with a low hydrophobic TMD [19], yet it is unknown how this complex recognizes proteins and exerts force to extract proteins from the IM.

MATERIALS AND METHODS

Strains and plasmids

Wild type *TIM17* or *TIM23* with its upstream endogenous promoter in pRS316 were transformed into W303-1α (*MAT* α, *ade2, can1, his3, leu2, trp1, ura3*) prior to genomic disruption of *TIM17* or *TIM23* gene with HIS3 gene through homologous recombination. Then, mutated *tim17* or *tim23* gene in pRS314 was transformed into the cell and FOA selection was followed to obtain mutant strains. Site-directed mutagenesis kit was used to generate *tim17* and *tim23* mutants and substituted residues are listed in Fig 2. *Δyta10* (*MAT* a *ade2-1, his3-11, 15, yta10::HIS3MX6, trp1-1, leu2, 112, ura3-52*) and W303-1a (*MAT* a, *ade2, can1, his3, leu2, trp1, ura3*) were used to test the involvement of the m-AAA protease in protein sorting. *Pam16-3* [22] was used as a control to check import ability of *tim23* mutant strains. All Mgm1p variants are in pHP84HA [21].

Isolation of mitochondria and protease protection assay

Mutant and wild type strains were grown in 1L of –His-Trp medium containing glucose (2% w/v) at 30°C until it reaches to 1-2O.D_{600} units per ml. Cells were collected by centrifugation at 3,000 *g* for 5 min and treated with 100mM of Tris base (pH 11.0) and 10mM Dithiothreitol (DTT), for 20 min at 30°C. Afterwards, cells were centrifuged at 2,000 *g* for 5 min and incubated with Zymolase-100T (5mg/g of cells) in 1.2M Sorbitol and 20mM K-phosphate at 30°C for 30 min (or up to 1hr). Cells were harvested by centrifugation at 1,200 *g* for 5 min at 4°C. Then, the pellet was resuspended in Homogenization buffer (10mM Tris-HCl, pH7.4, 1mM EDTA, 0.2% BSA, 1mM phenylmethanesulfonyl fluoride (PMSF), 0.6M Sorbitol), and cells were lysed by a glass-homogenizer at 4°C. In order to remove unbroken cells, the lysate was centrifuged at 1,200 *g* for 5 min. The mitochondrial fraction was obtained by centrifugation at 12,000 *g* for 15 min at 4°C. The pellet was resuspended in 500 μl of suspension buffer (0.6M Sorbitol, 20mM HEPES-KOH, pH7.4). 40μg of prepared mitochondria were incubated with 100μl of suspension buffer with or without Proteinase K (50μg/ml) for 30 min on ice. To inhibit the activity of Proteinase K, 1μl of 0.1M PMSF was added, and then the suspension was

incubated for 5 min on ice. Then, the samples were incubated for 15 min at 57°C with 50µl of sample buffer (50mM DTT, 1M Tris-HCl, pH7.6, 10% SDS, 30% glycerol, 0.5M EDTA, 100X protease inhibitor cocktail (PIC), 100mM PMSF) followed by SDS-PAGE and Western blotting analysis.

Pulse and chase

tim23 TMD2-1, *tim23 TMD2-2*, and its isogenic wild type cells were grown till it reaches between 0.3 and 0.8 $O.D_{600}$units per ml and 1.5 $O.D_{600}$units were collected by centrifugation at 3,000 *g* for 5 min. After washed twice with –Met liquid media, cells were resuspended in 1ml of –Met media and starved at 30°C or 37°C for 15 min and collected by centrifugation. After resuspension in 150ul of –Met, 5µl of [^{35}S]-Met were added and pulsed. Chase was conducted by supplying 50µl of cold Met (200µM) followed by incubation at 30°C or 37°C. Labeling was stopped by ice-cold buffer A (20mM Tris-HCl, pH 7.5, 20mM Sodium Azide). Harvested yeast cells were lysed in 100µl of Lysis buffer (20mM Tris-HCl, pH 7.5, 1% SDS, 1mM DTT, 1mM PMSF, protease inhibitor cocktail) and incubated at 65°C for 15 min. Cell debris was removed by centrifugation. Proteins were incubated with Agarose-G bead, anti-HA (mouse) antibody and IP buffer (15mM Tris-HCl, pH 7.5, 0.1% SDS, 1% Triton X-100, 150mM NaCl) for three hours in room temperature or overnight at 4°C. After washed with IP buffer, ConA buffer (500mM NaCl, 20mM Tris-HCl, pH 7.5, 1% Triton X-100), and Buffer C (50mM NaCl, 10mM Tris-HCl, pH 7.5), proteins were prepared by incubating with sample buffer (50mM DTT, 1M Tris-HCl, pH7.6, 10% SDS, 30% glycerol, 0.5M EDTA, 100X protease inhibitor cocktail (PIC), 100mM PMSF) for 15min at 60°C. Further analyses were conducted by SDS-PAGE and autoradiography (Fuji FLA-3000 phosphorimager, Fujifilm).

RESULTS

1. Mutagenesis of Tim17p and Tim23p

In order to understand the function of Tim17p and Tim23p in mitochondrial protein sorting, especially the role of each TMD, we have carried out site-directed mutagenesis of specific residues within these two subunits. With mutants that are viable, we conducted further studies. Growth phenotype of all generated mutants in this study is shown in Fig. 2. When both ends of the TMD2 of Tim23p were mutated, severe growth defect at 37°C was observed. Moreover, when the residue 149G (the matrix side of the TMD2) at Tim23p was mutated to W, I or E, mild growth defect at 37°C was detected.

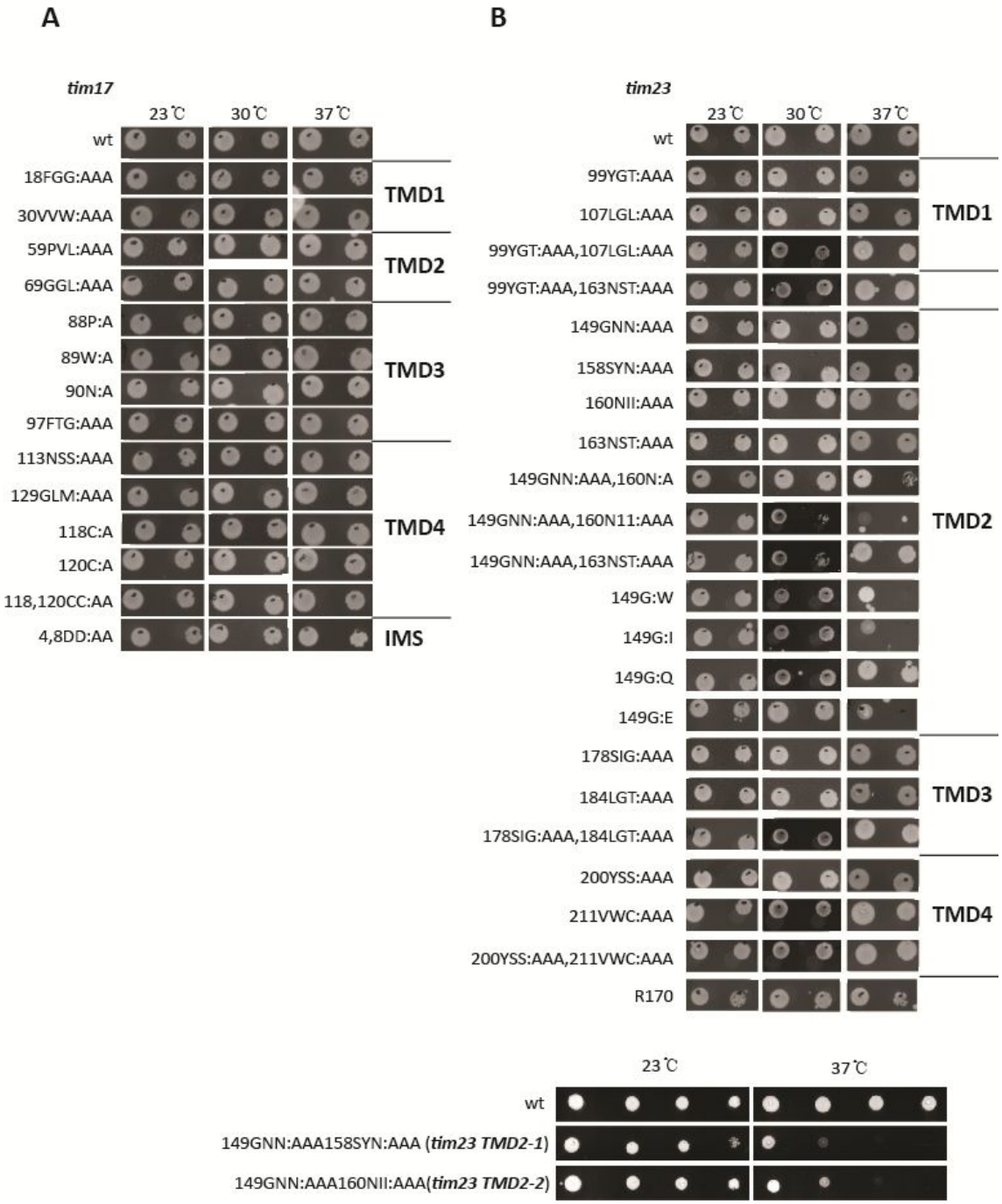


Fig. 3 Growth phenotype of *tim23* and *tim17* mutants All generated *tim17* (A) and *tim23* mutants (B) were grown overnight in –W media and diluted to OD_{600} 0.4. 100-fold diluted mutant cells were spotted alongside at –W plate containing glucose (2% w/v) and incubated under different temperature for two days. Growth phenotype of *tim23*149GNN:AAA158SYN:AAA (*tim23 TMD2-1*) and *tim23* 149GNN:AAA160NII:AAA (*tim23 TMD2-2*) were analyzed by 10-fold serial dilution (bottom).

2. Effects of tim17 mutants on the function of the TIM23 complex

All strains with a mutation at the end of each TMD of Tim17p were viable, except one with alanines near the IMS side of the TMD3, *tim17* 88PWM:AAA. Therefore, single alanine mutations at this site were generated for further study and their growth was tested along with other *tim17* mutants. The growth assay showed that none of the *tim17* mutants have growth defect at all temperatures (Fig. 3). To access whether these mutants also handle substrates in the same manner as the wild type Tim17p, we tested their ability of Mgm1p sorting (Fig. 4A, B). The ratio of *l*- and *s*-Mgm1p in the mutant strains was comparable to the wild type strain, indicating that these residues of Tim17p may not be critical for sorting of Mgm1p.

Two cysteine residues at the TMD4 were suggested to form a disulfide bond under respiring condition according to mass spectrometry data (personal communication with Joseph Kwon, in KBSI). To examine whether the formation of disulfide bond between these two residues is crucial for Tim17p function in non-fermentable carbon source, they were mutated to alanines individually or simultaneously. The Cys mutant *tim17* strains expressing Mgm1p were grown in either fermentable (glucose containing media) or respiring (glycerol containing media) conditions and sorting of Mgm1p was analyzed. Despite a lack of the disulfide bond in Tim17p, its capability to properly sort Mgm1p was not altered (Fig. 4C).

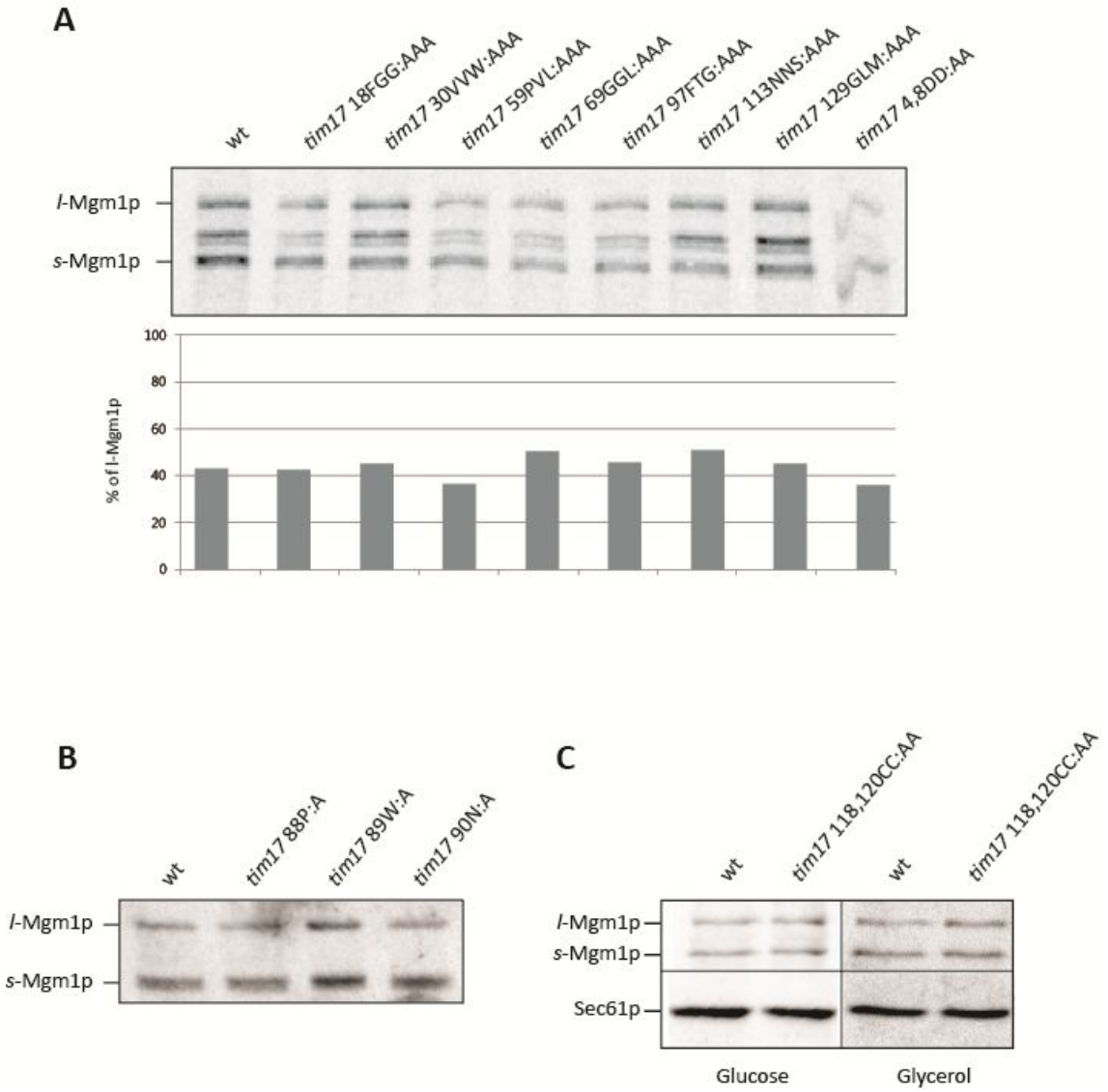


Fig. 4 Mgm1p sorting in *tim17* mutant strains Mgm1p with C-terminal HA tag was expressed in *tim17* mutant and the isogenic *TIM17* wild type strains. (A) Mgm1p in mutant strains, harboring 3 alanines at one end of each TMD of Tim17p, was labeled with ^{35}S-Met for 5 min and analyzed by SDS-PAGE and autoradiography. Ratio of *l*- and *s*-Mgm1p was quantified. (B) Sorting of Mgm1p in *tim17* mutant, with single alanine at the IMS side of TMD3, was tested by Western blot analysis. (C) *tim17* 118,120CC:AA mutant and the isogenic *TIM17* wild type cells expressing HA tagged Mgm1p were grown at glucose (2% w/v) or glycerol (3% w/v) containing –L-W media overnight at 30°C. Mgm1p was detected by anti-HA antibody. As a loading control, Sec61p was detected by anti-Sec61p antibody.

3. Effects of tim23 mutants

3.1. Mutations in both ends of the TMD2

3.1.1. Mutagenesis in both ends of the TMD2 leads to accumulation of precursor proteins

Among *tim23* mutants generated from this study, those that were substituted with 3 alanines at both ends of the TMD2 (149GNN:AAA158SYN:AAA, 149GNN:AAA160NII:AAA) showed growth defects at non-permissive temperature, indicating that these residues are indispensable for proper function of Tim23p (Fig. 3B). We reasoned that these mutations might have caused mis-localization of Tim23p, an altered interaction with other components of the complex, or defects in import and membrane insertion of proteins. We further analyzed the effects of *tim23* 149GNN:AAA158SYN:AAA and *tim23* 149GNN:AAA160NII:AAA on the function of the TIM23 complex. For convenience, these *tim23* mutants will be referred as *tim23 TMD2-1* and *tim23 TMD2-2*, respectively.

First, to examine whether these mutants are correctly targeted to the mitochondrial IM, protease protection assay was carried out with the isolated mitochondria from yeast strains expressing mutated Tim23p. Mutated Tim23p was protected from protease K, while the OM protein, Tom70p was degraded, indicating these mutants were properly targeted and localized in the IM. Thus, incorrect localization of the mutated Tim23p could be ruled out (Fig. 5A). To investigate how these *tim23* mutants handle protein import and sorting, we have employed various substrates. To begin with, sorting of Mgm1p was analyzed by pulse labeling and autoradiography in yeast strains, which harbor mutations at the TMD2 of Tim23p. The ratio of *l*- and *s*-form of Mgm1p shows approximately 1:1 in the wild type strain, while the *l*-form of Mgm1p was largely increased in *tim23 TMD2-1* and *tim23 TMD2-2*. The proportion of laterally released Mgm1p was slightly more increased in *tim23* 149GNN:AAA160N:A, compared with other mutants that have triple alanines at only one side of the TMD2, but not as significantly as *tim23 TMD2-1* and *tim23 TMD2-2* (Fig. 5B). In an attempt to reveal the reason why these mutants with alanines at both ends of the TMD2 yield more *l*-Mgm1p, we closely examined the process by pulse and chase experiments. Interestingly, precursor form (*) was detected first in *tim23 TMD2-2* strain and *l*-Mgm1p was slowly appeared over time (Fig. 5C).

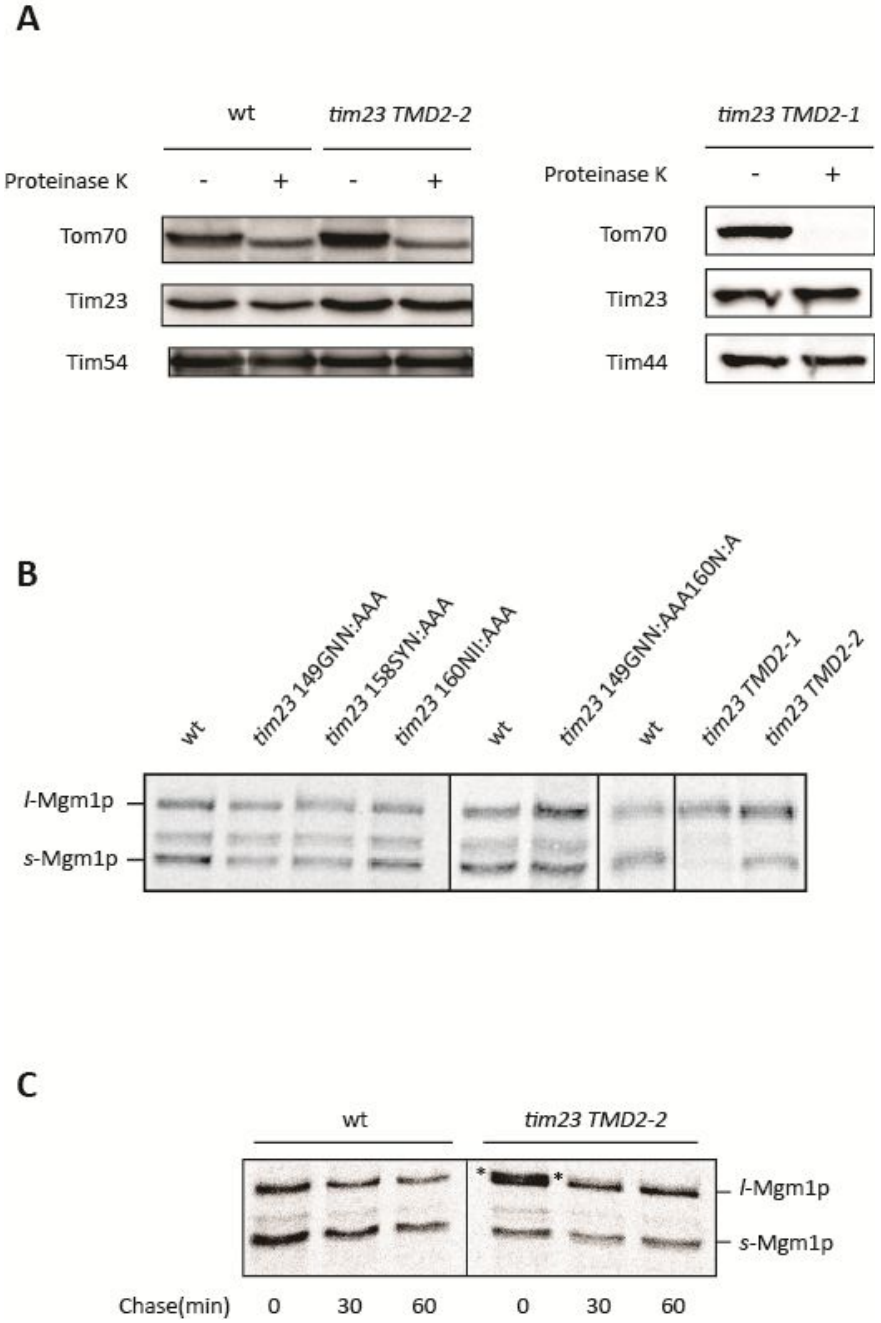


Fig. 5 Effects of Tim23p mutation at both ends of the TMD2 on its localization and Mgm1p sorting (A) Mitochondria were isolated from *tim23 TMD2-1*, *tim23 TMD2-2*, and the isogenic *TIM23* wild type cells and incubated with either 100μl of suspension buffer or suspension buffer with Proteinase K (50μg/ml) for 30 min on ice. After SDS-PAGE, indicated proteins were detected as a marker for different mitochondrial subcompartments. (Tom70; outer membrane, Tim54; inner membrane, Tim44; matrix) (B) Strains with Tim23p mutation at the TMD2 and wild type Tim23p expressing Mgm1p-HA were radiolabeled for 5 min at 37℃ followed by SDS-PAGE and autoradiography. (C) *tim23 TMD2-2* and the wild type cells expressing Mgm1p-HA were pulse-labeled and chased for indicated time at 37°C. Asterisks (*) indicate bands corresponding to the precursor form of Mgm1p.

To further dissect the mechanism, we have studied the import capacity of the mutants with a matrix targeted protein, Hsp60p. When grown at non-permissive temperature, the precursor form of Hsp60p was detected in the mutant strains, showing decreased efficiency of import with the mutants. It is not likely that the mutants lose import capacity under heat stress completely, like *pam16-3*, since mature form of Hsp60p was observed (Fig. 6A). We could also detect both accumulated precursor form and imported mature form of Hsp60p by pulse experiment in *tim23 TMD2-1* (Fig. 6B). These results indicate that the import ability of *tim23 TMD2-1* is partially impaired.

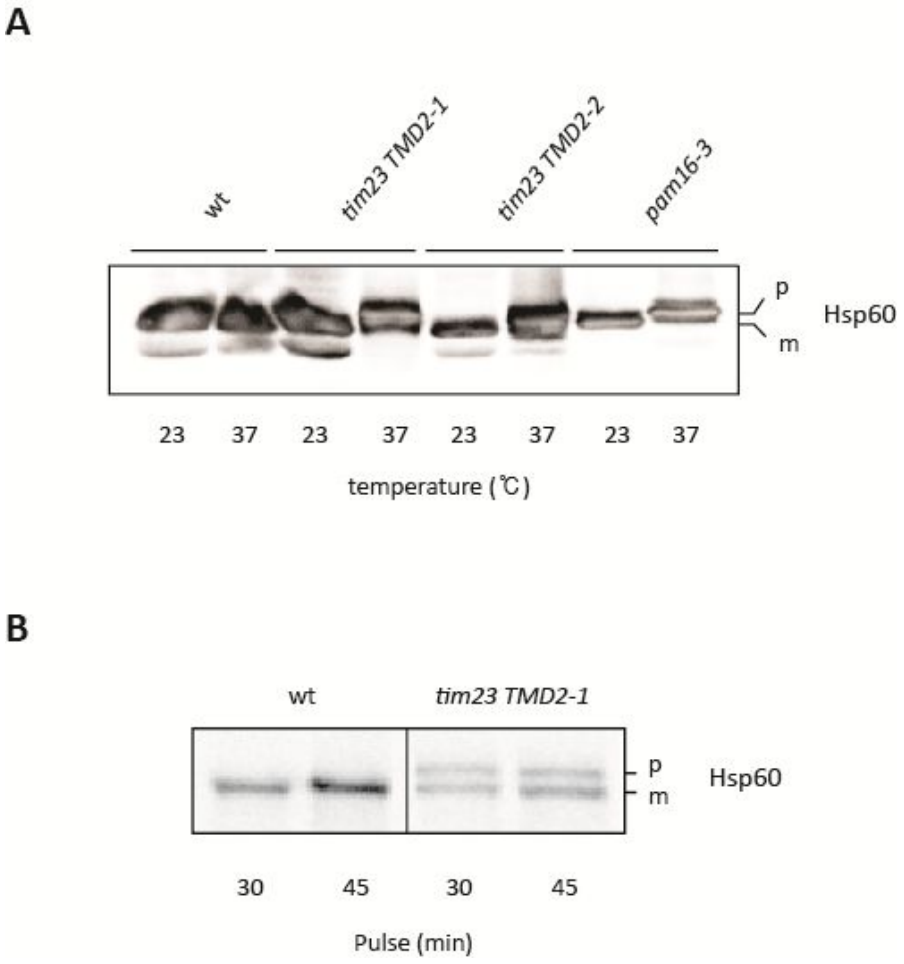


Fig. 6 Tim23p mutation at both ends of the TMD2 leads defect in import of matrix targeted Hsp60p (A) *tim23* mutants and *pam16-3* were grown at 23°C overnight and further incubated either at 23°C or 37°C for 12hrs. Endogenous Hsp60p was detected by Western blot analysis. (B) *tim23 TMD2-1* and the isogenic *TIM23* wild type cells were radiolabeled with ^{35}S-Met for indicated times at 37°C. Endogenous Hsp60p from both strains was immunoprecipitated by anti-Hsp60p antibody and analyzed by SDS-PAGE and autoradiography. (p; precursor form of Hsp60p, m; mature form of Hsp60p)

To corroborate this finding, we used Mgm1p variants whose first TMD is imported to the matrix side, 19A and Mba1 MFP (Mgm1 Fusion Protein). 19A is an Mgm1p with its first TMD substituted with a 19 alanine stretch [21] (Fig. 7A). Since the first TMD of this construct is less hydrophobic, it is imported to the matrix and yields mainly *s*-Mgm1p in wild type cell. Mba1p is an IM protein which follows conservative sorting pathway; therefore it is fully imported to the matrix by the TIM23 complex prior to membrane integration by the OXA1 complex. For this reason, fusing the C-terminal portion of Mgm1p to the N-terminal portion of Mba1p is expected to yield mainly *s*-form [22] (Fig. 7B). If *s*-form of these constructs is generated in *tim23* mutant strain, to the comparable level of wild type cells, this reflects proper import ability. When pulsed and chased, both 19A and Mba1 MFP were accumulated as a precursor form and slowly imported and generated *s*-Mgm1p in both *tim23 TMD2-1*and *tim23 TMD2-2*. This result strengthens that the *tim23* mutant has defective import ability.

Lastly, substrates, which have flanking charges or prolines in their TMD, were utilized to test how *tim23 TMD2-1* affect sorting of these proteins [21] (Fig. 7C). In all substrates, precursors were detected in the mutant after 5 min labeling. These results indicate that *tim23 TMD2-1* causes accumulation of precursor proteins regardless of the sequence context of proteins.

In conclusion, mutations in both ends of the TMD2 of Tim23p partially impair the import of substrates, thus proteins were imported slowly. This observation shows that the TMD2 of Tim23p is important for protein sorting.

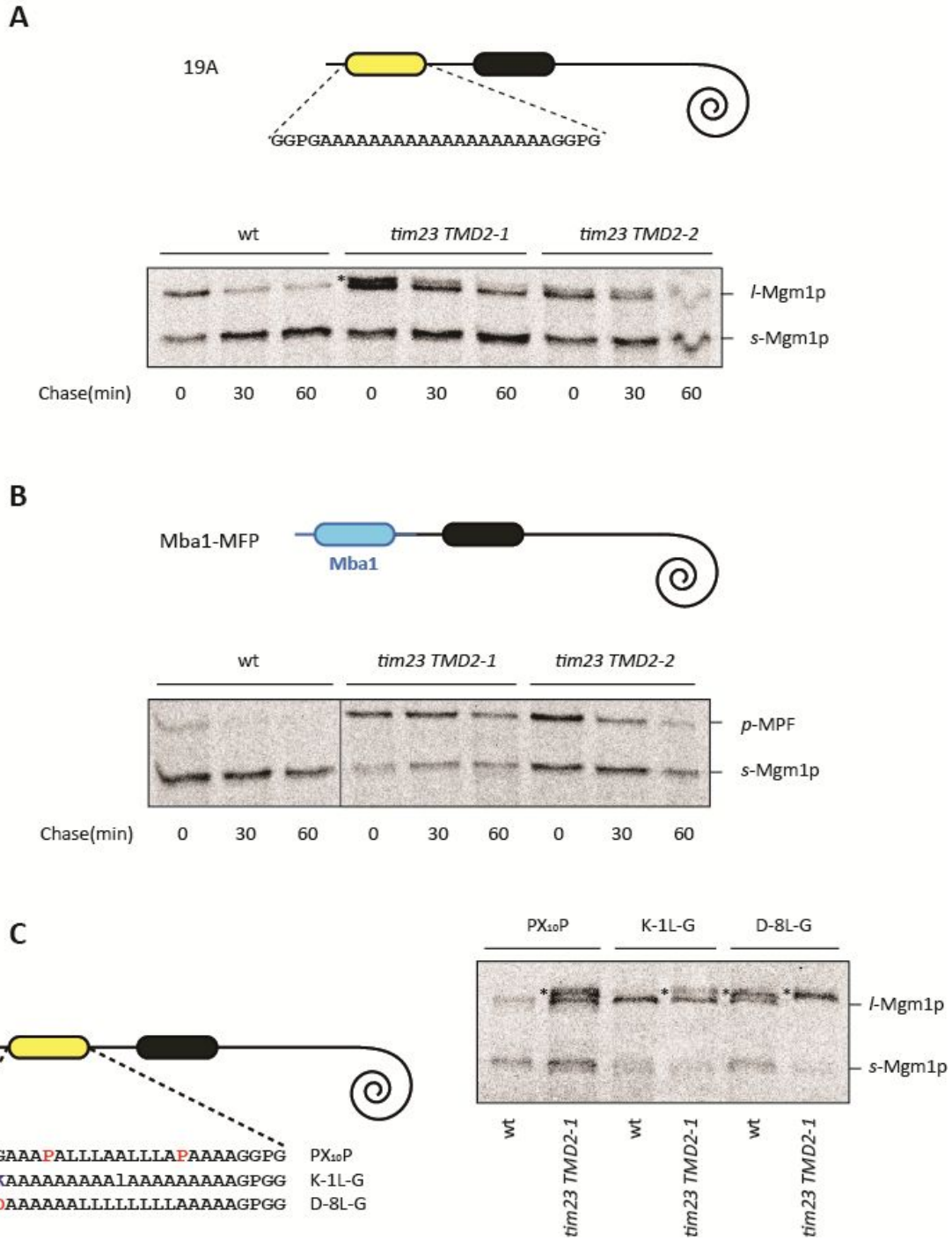


Fig. 7 Tim23p mutation at both ends of the TMD2 causes preprotein accumulation *tim23* mutants and wild type cell expressing 19A (A) and Mba1 MFP (B) were radiolabeled for 5 min and chased for indicated time at 37°C. (C) $PX_{10}P$, K-1L-G, and D-8L-G Mgm1p variants in *tim23 TMD2-1* and the isogenic wild type cells were labeled for 5 min at 37°C. TMD1 sequence of each constructs is presented. (Asterisk* and *p*-MFP; precursor form)

3.2. Mutation at the matrix side of the TMD2

3.2.1. Mutation at the matrix side of the TMD2 of Tim23p increases lateral insertion of Mgm1p

Increasing evidence indicates that the TMD2 of the Tim23p is crucial for substrate sorting. Fluorescence mapping and cross-linking studies indicate that the hydrophilic side of the TMD2 interacts with incoming substrates [9, 10]. Indeed, among the *tim23* mutants that we generated, one with mutations at both ends of the second TMD (*tim23 TMD2-1* and *tim23 TMD2-2*) showed impaired import and accumulation of precursor proteins. To narrow down a specific site crucial for this effect, we generated more mutations at the TMD2 of Tim23p and found a single residue mutation that affected the sorting of mitochondrial IM proteins. When the 149G of Tim23p (near the matrix side the TMD2) was mutated to tryptophan, growth was decreased at 37°C (Fig. 3B) and when Mgm1p was expressed in this mutant, more *l*-Mgm1p was detected by Western blot analysis (Fig. 8A). In order to distinguish whether it was the specific replacement of G to W at this site that caused the increased *l*-Mgm1p formation, or the maintenance of G at this location is important, different residues (I, Q and E) were substituted in this site. Changing the G to polar glutamine did neither show growth defect at 37°C nor increased ratio of *l*-Mgm1p. Replacement to a hydrophobic residue, Isoleucine showed both slight growth defect at elevated temperature and increase of *l*-Mgm1p, but it was not substantial as 149G:W. When 149G was substituted with a negatively charged glutamate, *l*-Mgm1p was increased to the comparable level to 149G:W (Fig. 8A).

3.2.2. Import capability of tim23 149G:W is not impaired

Increased ratio of *l*-Mgm1p over *s*-Mgm1p in *tim23* 149G:W might have stemmed from import defect as in the *tim23 TMD2-1* and *tim23 TMD2-2*. To test import efficiency of *tim23* 149G:W, sorting of Hsp60p was studied (Fig. 8B). Unlike *tim23 TMD2-1*, accumulation of precursor form of Hsp60p was not observed with *tim23* 149G:W, indicating that the import ability of *tim23* 149G:W is intact.

To further support that the import capability of this mutant is sound, Mba1 MFP and Cox18(1TM) MFP were tested. Like Mba1, Cox18 is a conservatively sorted protein, therefore when fused to the C-terminal part of Mgm1p, *s*-form is generated in the wild type cell.

Formation of *s*-form with these constructs in the *tim23* mutant strain would thus reflect proper import ability. The first TMD of Mba1 MFP and Cox18(1TM) MFP were imported to the matrix side and yielded predominantly *s*-Mgm1p in both wild type and *tim23* 149G:W (Fig. 8C). This observation reinforced that *tim23* 149G:W mutant did not interfere with general import.

Proper import of Hsp60p to the matrix in *tim23* 149G:W implies that the interaction of the mutated Tim23p with the PAM motor is maintained. To strengthen this connotation, a substrate that is partitioned to the IM in a PAM independent manner, Cyt1 MFP, was used [22] (Fig. 8D). Similar to Mgm1p, which sorted PAM-dependently, *l*-form of Cyt1 MFP was increased in *tim23* 149G:W. These results suggest that enhanced membrane insertion of tested substrates did not stem from altered interaction between the TIM23 complex and the PAM motor, thus ruled out the possibility that mutations in 149G:W in Tim23p might impair dynamic interaction with the PAM complex.

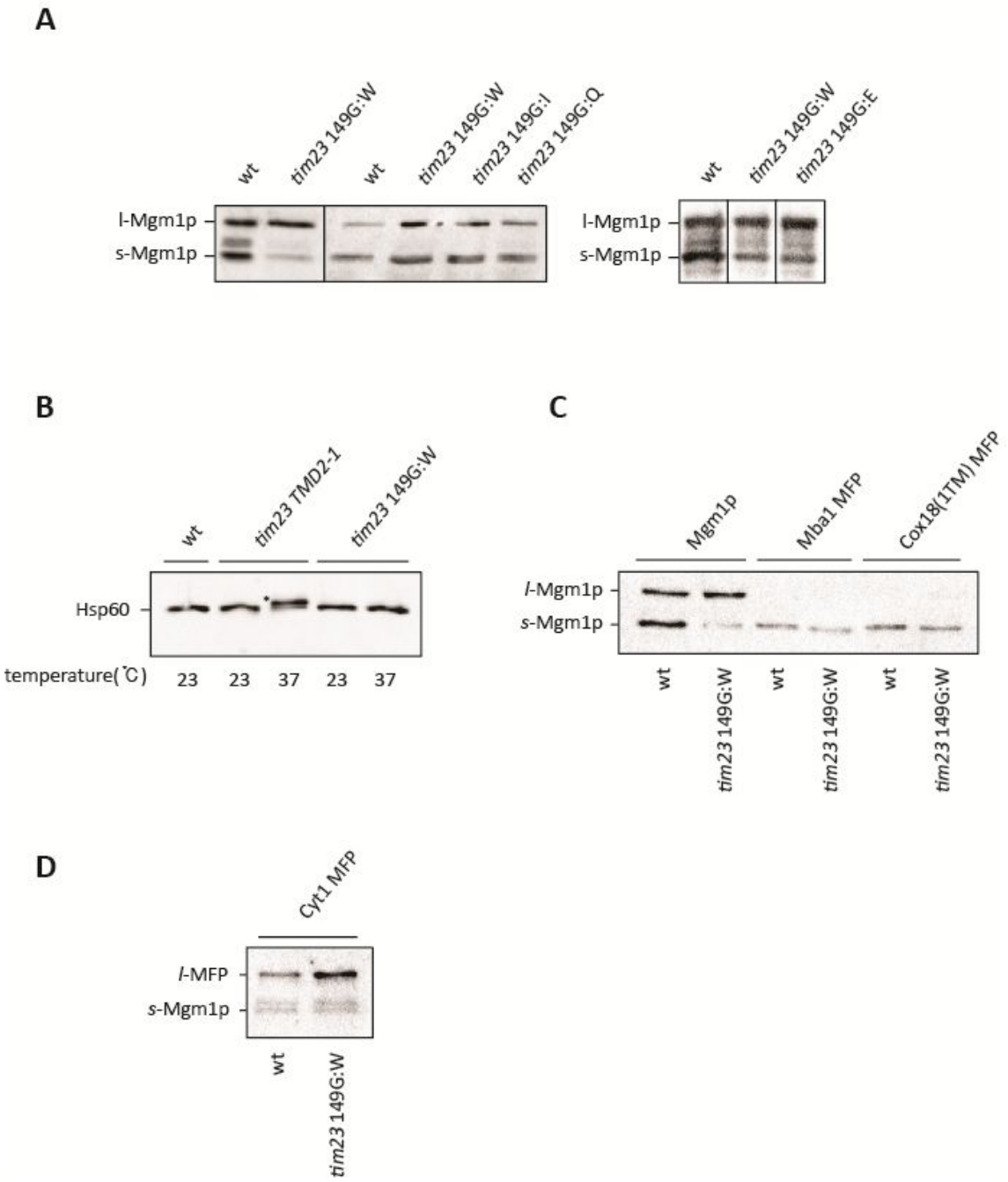
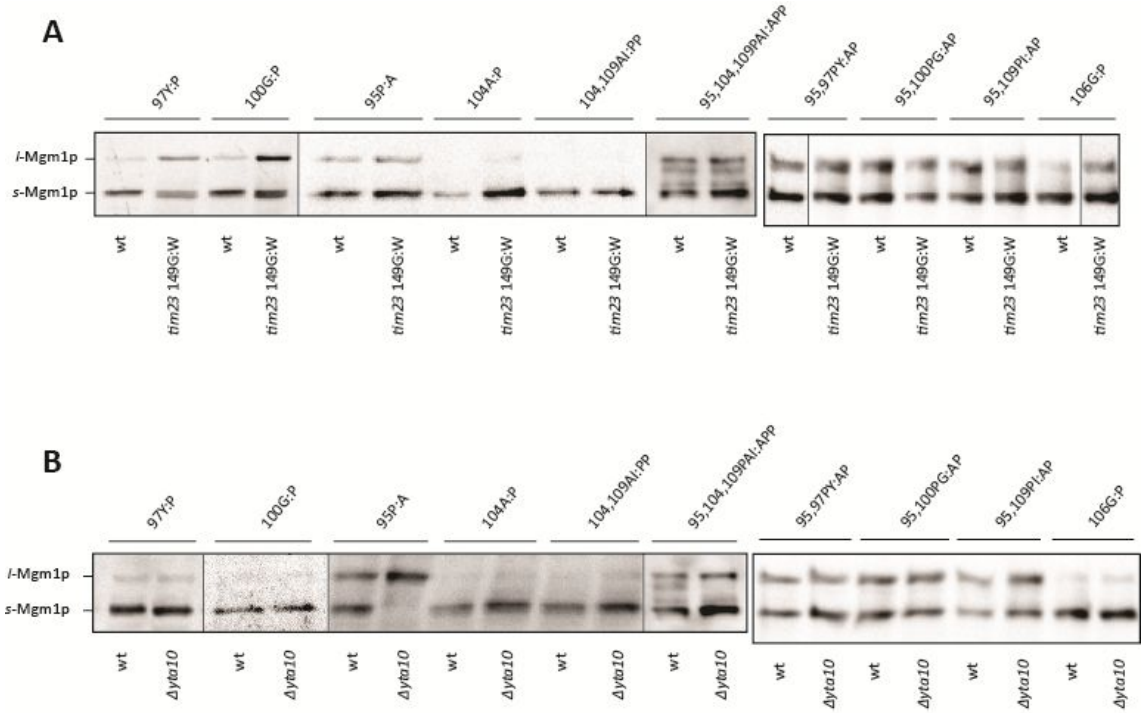


Fig. 8 Effects of mutation at Tim23p 149G in protein sorting (A) Mgm1p-HA was transformed to *tim23* mutant strains, with varying substitution at residue 149. After grown at 30°C overnight, sorting of Mgm1p in each strain was analyzed by Western blotting. (B) *tim23* mutant strains were grown in glucose (2% w/v) containing –W media for overnight at 23°C and further grown at 23°C or 37°C for 12 hrs. Expression of endogenous Hsp60p in each strain was analyzed by Western blotting with anti-Hsp60p antibody (C) Mgm1p and MFPs were introduced to *tim23* 149G:W and the isogenic *TIM23* wild type strain and their sorting were accessed by Western blot analysis. (D) *tim23* 149G:W and wild type cells expressing Cyt1 MFP were grown at 30°C and the ratio of *l*- and *s*- form of Cyt1 MFP was analyzed by Western blotting.

3.2.3. Sequence characteristics of substrates that were more membrane inserted in tim23 149G:W To further characterize how *tim23* 149G:W mutant handles substrates, Mgm1p variants with diverse sequence contexts within the first TMD were analyzed. It has been shown that having proline residue within the TMD renders membrane insertion [21]. To determine whether *tim23* 149G:W enhances membrane integration of the TMD with proline residues, a set of Mgm1p variants with proline residues with varying positions in the first TMD were tested (Fig. 9C). Elevated *l*-Mgm1p in *tim23* 149G:W strain was detected when there were more than 2 proline residues at the N-terminal side of Mgm1p (Fig. 9A).

It was demonstrated that the m-AAA protease can dislocate proteins from the inner membrane [19]. If these Mgm1p variants with prolines are recognized and pulled by the m-AAA complex after insertion, we may not be able to detect the effect of *tim23* 149G:W by Western blotting, which represent a steady-state level of proteins, even if more *l*-Mgm1p were generated by the mutant. To exclude this possibility, the same set of constructs was also tested in the m-AAA protease defective strain (Fig. 9B). However, none of the constructs, apart from 95P:A, was influenced by the m-AAA protease. This confirms that the effect shown in Fig. 9A is attributable to *tim23* 149G:W mutant.



C

Mgm1p variants with P	Sequence of the TMD1	Pulled by the m-AAA protease	*l* -Mgm1p increased in *tim23* 149G:W
WT	IIRLPIYVGGGMAAAGSYIAYKM	–	+
97Y:P	IIRLPIPVGGGMAAAGSYIAYKM	–	+
100G:P	IIRLPIYVGPGMAAAGSYIAYKM	–	+
95P:A	IIRLAIYVGGGMAAAGSYIAYKM	+	–
104A:P	IIRLPIYVGGGMAPAGSYIAYKM	–	–
104,109AI:PP	IIRLPIYVGGGMAPAGSYPAYKM	–	–
95,104,109PAI:APP	IIRLAIYVGGGMAPAGSYPAYKM	–	–
95,97PY:AP	IIRLAIPVGGGMAAAGSYIAYKM	–	–
95,100PY:AP	IIRLAIYVGPGMAAAGSYIAYKM	–	–
95,109PI:AP	IIRLAIYVGGGMAAAGSYPAYKM	–	–
96G:P	IIRLPIYVGGGMAPAGSYIAYKM	–	–

Fig. 9 Sorting of Mgm1p with prolines in *tim23* 149G:W (A) Mgm1p constructs having P in its TMD1 were expressed in *tim23* 149G:W and the isogenic *TIM23* wild type strain. Their sorting was analyzed by Western blotting with anti-HA antibody. (B) Mgm1p constructs with P were transformed to *Δyta10* (*MAT* a *ade2-1, his3-11, 15, yta10::HIS3MX6, trp1-1, leu2, 112, ura3-52*) and W303-1a (*MAT* a, *ade2, can1, his3, leu2, trp1, ura3*). Whole-cell lysates were analyzed by Western blotting. (C) TMD1 sequence of Mgm1p variants with P.

Mgm1p contains charged residues flanking the first TMD and it is shown that these charged residues play a decisive role for the insertion of this protein [23]. When these flanking charged residues in Mgm1p were mutated to alanines or to the opposite charges, insertion was disfavored, mostly generating *s*-Mgm1p. We tested whether *tim23* 149G:W mutant can rescue insertion of these Mgm1p variants (Fig. 10). All Mgm1p with single positive charged residue mutation showed increased insertion in the *tim23* mutant, except for Mgm1p variants, 79R:A and 78R:D. Previously, it was postulated that 79R of Mgm1p is a strong determinant for membrane insertion because 79R:A was imported even without a functional import motor [23]. Furthermore, negative charges, such as 78R:D, near the matrix side has been shown to be unfavorable due to the membrane potential built across the IM. For these reasons, 79R:A and 78R:D may harbor a strong feature for import that can overcome the effect of the *tim23* 149G:W. *l*-Mgm1p was not increased in the *tim23* mutant with Mgm1p variants, 78,79RR:AA and 78,79RR:AA GMM:VVL, whilst insertion of 78,79RR::KK enhanced. Together, these results confirm that a positive charge at position 79 in Mgm1p is indeed a significant influence for membrane insertion. When more than 2 positive residues flanking N-terminus of TMD1 of Mgm1p were mutated, like 86,90,93KRR:AAG and 90,93KR:AA, membrane insertion was not raised in the *tim23* 149G:W. This observation indicates positively charged N-terminal flanking residues of the Mgm1p are strong sequence determinants for membrane integration, thus when mutated, these constructs could not be inserted even in the *tim23*149G:W mutant. On the other hand, when negatively charged flanking residues at the IMS side were replaced to alanines or positively charged lysines, 114,115EE::AA and 114,115EE::KK respectively, membrane insertion of Mgm1p was increased in the *tim23* mutant. This implies that negatively charged flanks at the IMS side is not a cogent criterion as positively charged flanks at the matrix side for the membrane partitioning of the first TMD of Mgm1p. Moreover, together with the observation that the *tim23* 149G:W mutant did not interrupt the import of matrix targeted proteins and conservatively sorted proteins (Fig. 8), it is likely that the *tim23* 149G:W mutant does not enhance membrane insertion of proteins if they have strong import characters, but rather improves insertion of proteins that are relatively stable in the IM. As for the Mgm1p variants with proline residues, the m-AAA protease dependence of these constructs was accessed and,

except 78,79RR:AA GGM:VVL, none of them were dislocated by the m-AAA complex (Fig. 10B). This confirms that the observed effects are caused by the *tim23* 149G:W and does not involve the m-AAA protease.

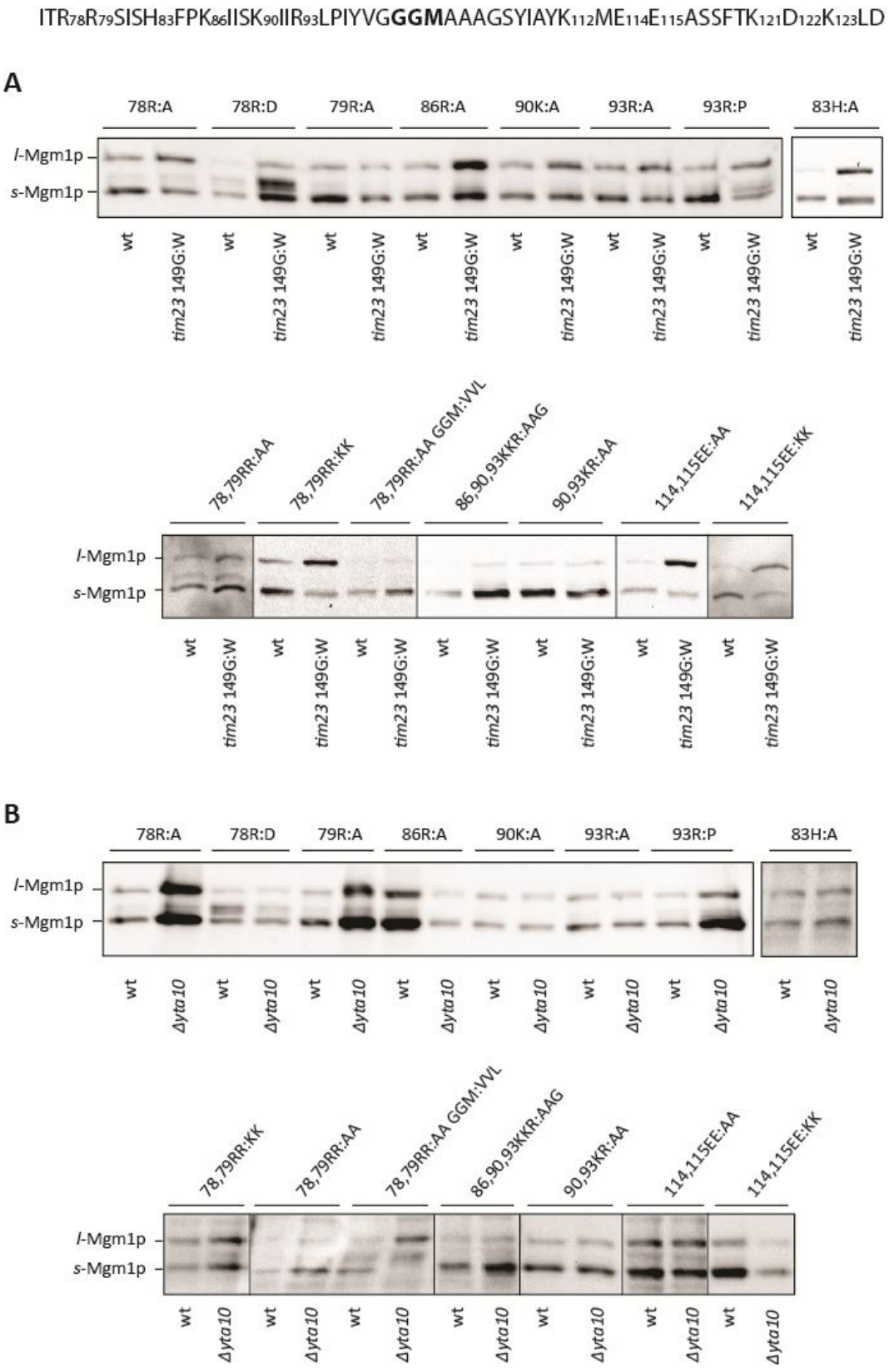


Fig. 10 Sorting of Mgm1p with altered flanking charges in *tim23* 149G:W Mgm1p variants with altered flanking charges were transformed to *tim23* 149G:W (A), *Δ yta10* (B) and their isogenic wild type cells. Whole-cell lysates were analyzed by Western blotting. Sequence of the Mgm1p (from residue 76 to residue 125) is presented above.

3.2.4. Effects of tim23 149G:W at the early stage of mitochondrial protein sorting

We showed that *tim23* 149G:W increases membrane insertion of proteins that are relatively stable in the IM, like an Mgm1p variant, 78,79RR:KK, but not those with a strong import character, such as 78,79RR:AA. This result was observed in steady state level of the substrates. To survey whether *tim23* 149G:W inserts the TMD of 78,79RR:AA better from the early point of its biogenesis, pulse and chase experiment was carried out. 78,79RR:KK shows more *l*-Mgm1p in 149G:W from the 0 min time point during the chase, indicating that the *tim23* mutant raises *l*-Mgm1p formation from the early stage of protein sorting. Surprisingly, 78,79RR::AA, of which steady state level of *l*-and *s*-Mgm1p ratio were not affected by the *tim23* mutant, showed more *l*-form in the mutant at the early point compared to the wild type (Fig. 11A). It is probable that *tim23 149G:W* holds incoming substrates at the translocon for a prolonged time, but depending on the feature of substrates, some are membrane inserted and others are imported eventually.

3.2.5. Effects of tim23 149G:W on membrane potential

Recently, it has been reported that membrane potential of mitochondrial IM was disrupted when 149G of Tim23p was replaced by E [24]. This new finding made us wonder whether *tim23* 149G:W also reduces membrane potential. To find out whether impairment of membrane potential occurs in *tim23* 149G:W strain, sorting of Mgm1p was tested in *tim23* 149G:W grown either in fermentable or respiring conditions (Fig. 11B). While yeast cells in fermentable carbon source preferentially generate ATP by substrate-level phosphorylation, energy metabolism of yeast cells using non-fermentable carbon source is largely dependent on the respiratory chain complex. Therefore, more membrane potential is built in respiring condition. In case of wild type strain, the ratio of *l*- and *s*-Mgm1p is the same in both conditions. On the contrary, *s*-Mgm1p was increased in *tim23* 149G:W under respiring condition. This may indicate that *s*-Mgm1p generation was decreased in *tim23* 149G:W in fermentable condition, possibly due to reduced membrane potential. However, when membrane potential was restored to some extend by inducing respiration, increased portion of the first TMD of Mgm1p was imported, lessening the mutant phenotype.

In order to consolidate this hypothesis, substrates whose *l*-form increased in *tim23*

149G:W and those not affected by the mutant were tested in *tim23* 149G:E strain (Fig. 11C). In Fig. 9 and 10, we observed that the ratio of *l*-Mgm1p in *tim23* 149G:W was increased when positive charges or 2 prolines were present at the N-terminal end of Mgm1p first TMD (78,79RR:KK and 100G:P, respectively). Whilst, enhanced *l*-Mgm1p generation was not detected with Mgm1p that has neutral residues at the N-terminal end the TMD (78,79RR:AA) or prolines near the C-terminal end of the TMD (95,104,109PAI:APP) in *tim23* 149G:W mutant. Therefore, we chose these four constructs to test in *tim23* 149G:E, to see whether *tim23* 149G:E affects protein sorting with the same pattern observed in *tim23* 149G:W. As in *tim23* 149G:W, *l*-Mgm1p was increased with Mgm1p variants, 78,79RR:KK and 100G:P, but not with 78,79RR:AA and 95,104,109PIA:APP in *tim23* 149G:E. Similar phenotypes of *tim23* 149G:W and *tim23* 149G:E in handling of the substrates have been observed, thus these results suggest that increased membrane insertion of proteins in these mutants may be due to weakened membrane potential across the inner membrane.

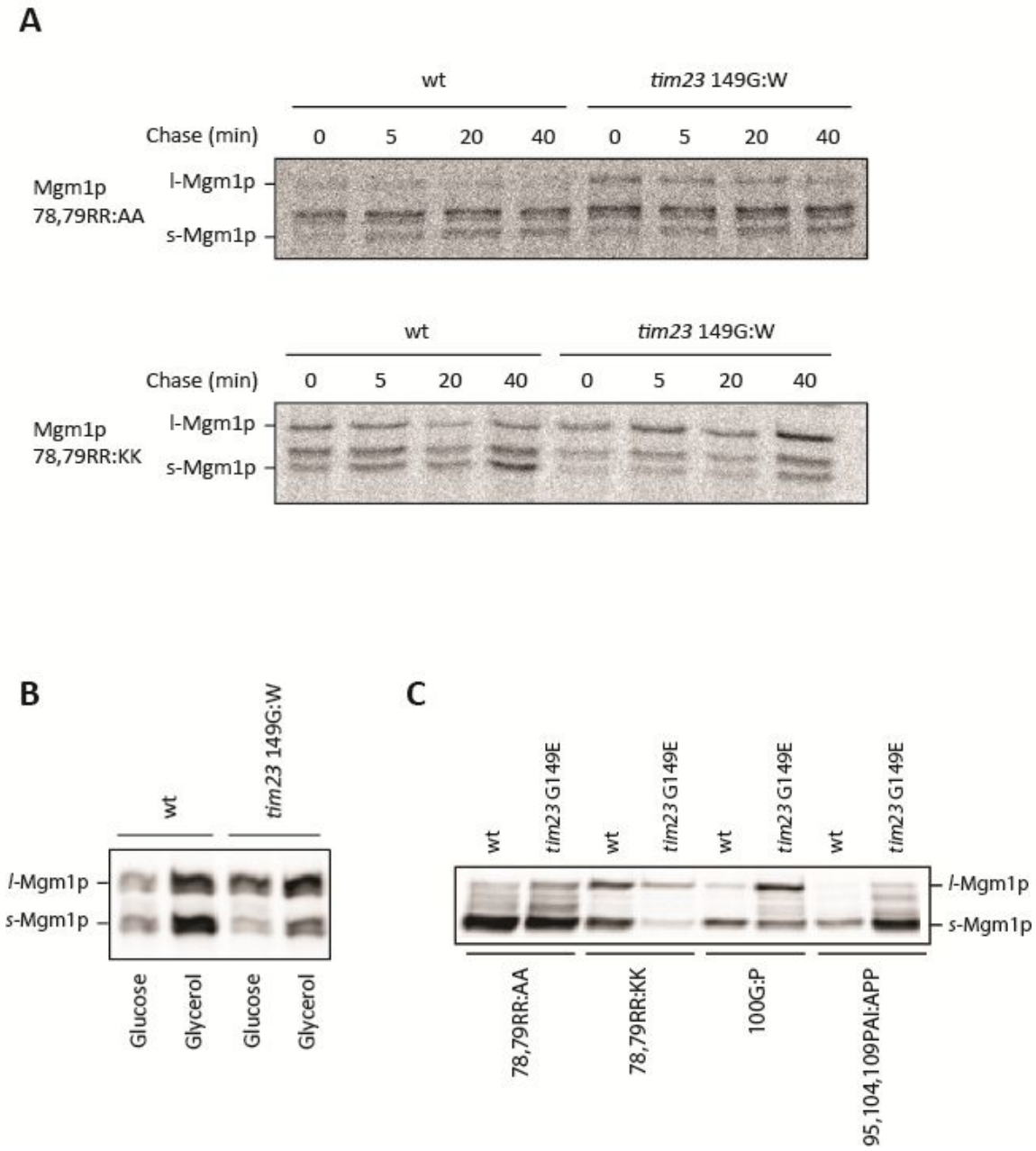


Fig. 11 *tim23* 149G:W affects protein sorting from the early stage and this phenotype may be caused by reduction in membrane potential (A) *tim23* 149G:W and wild type cells expressing Mgm1p variants (Mgm1p 78,79RR:AA, Mgm1p 78,79RR:KK) were radiolabeled for 5min and chased for indicated time, followed by SDS-PAGE and autoradiography. (B) *tim*23 149G:W and the isogenic TIM23 wild type cells expressing Mgm1p were grown at glucose (2%) or glycerol (3%) containing media and Western blot analysis was conducted. (C) Sorting of Mgm1p variants were analyzed in *tim23* 149G:E.

4. Characteristics of proteins recognized by the m-AAA protease

While testing Mgm1p variants in *tim23* mutants, we noticed that there are some variants whose *s*-form formation is influenced by the m-AAA protease, such as 95P:A and 78,79RR:AA GMM:VVL (Fig 9B and 10B). Previous study showed that Mgm1p with less hydrophobic TMD are dislocated from the IM by the m-AAA protease [19]. To further elucidate what properties of TMDs are recognized by the m-AAA complex for dislocation, sets of Mgm1p variants harboring P, K and D at different positions along the first TMD were tested in the m-AAA defective, *Δyta10* strain.

Mgm1p variants with single P mostly produced *l*-Mgm1p in the absence of the m-AAA subunit compared to the wild type strain (Fig. 12A). These results suggest that they were laterally released to the IM at the level of the TIM23 complex but subsequently dislocated by the m-AAA complex. Depending on the position of the P within the TM segment, the m-AAA acts on differently. When P was in the middle of the TM segment, more Mgm1p were pulled by the m-AAA complex than when the P was at either ends of the TMD (Fig. 12A). Effects of P in membrane insertion and dislocation were further tested with Mgm1p variants having symmetrically disposed pair of prolines from the middle towards the ends of the TMD (Fig. 12B). Unlike the constructs with single P, these proteins formed more *s*-Mgm1p at the level of the TIM23 complex, indicating that the TIM23 complex imports these variants to the matrix. But among the populations that were membrane inserted, they were still pulled by the m-AAA protease. Positional dependence of m-AAA complex recognition was observed with double P variants. When a pair of P is located in the middle of the TMD, more *l*-Mgm1p were observed compared to that two Ps are present further apart in the TMD in the m-AAA defective strain, indicating that the m-AAA is sensitive to the positions of P in the TMD. When two Ps are present in the middle, it causes a kink in the middle of the TMD whereas if the two Ps are further apart, it causes the TMD to contain two kinks along the TMD. When prolines are present at the ends of Mgm1p TMD, more *l*-Mgm1p were generated compared to that two Ps scattered within the TMD, indicating that these Mgm1p variants were dislocated lesser extent. In sum, these data show that presence of P in the TMD is a strong determinant for the m-AAA dislocation activity and the m-AAA protease senses differently depending on positions of P

within the TMD. Thus, these results suggest that perturbation of an alpha helix formation within the TMD may be recognized by the m-AAA protease and triggers its dislocation activity.

Effects of charged residues in membrane insertion by the TIM23 complex and dislocation by the m-AAA protease was examined with single K and D scans in the first TMD of Mgm1p (Fig. 12C). When a charged residue, K or D, was in the middle of the TMD (position 6 to 14), *s*-Mgm1p was detected in *Δyta10* strain. This result shows that the TIM23 complex can mediate their import to the matrix in absence of the functional m-AAA protease. But, some populations that were inserted to the membrane, further dislocated by the m-AAA protease. When a charged residue was at either end of the TMD, only *l*-Mgm1p was observed in *Δyta10* strain. This indicates that the TIM23 complex laterally releases Mgm1p to the membrane if it has a charged residue flanking the TMD. However, in the presence of the m-AAA protease, *s*-form of these substrates was appeared in different extent. When a negative charged residue was at the matrix side of the TMD, greater amount of *s*-Mgm1p was generated by the m-AAA protease, compared to the substrates that contain a negative charged residue at the IMS side or a positive charged residue at either end of the TMD. If negative charges are found near the matrix side, such TMDs become more sensitive to the m-AAA pulling, possibly due to unstable state of these proteins in the IM. Taken together, these results suggest that the m-AAA protease may exert its dislocation activity in greater extent if proteins that are integrated by the TIM23 complex, are instable in the IM, for instance, by having negative charges at the matrix side.

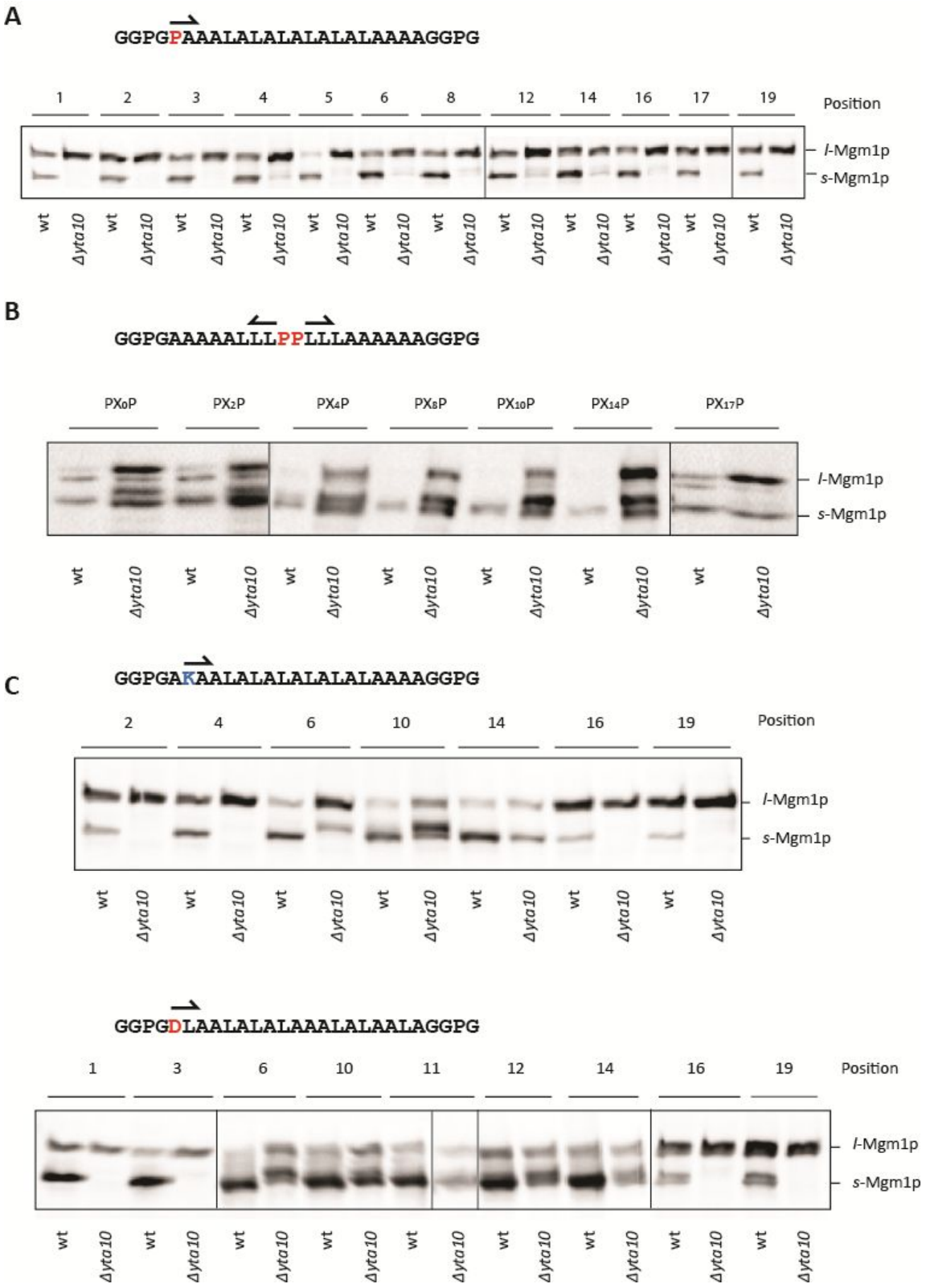


Fig. 12 Positional dependence of P, K, D recognition by the m-AAA protease Mgm1p variants, with single P (A), double P (B), K and D(C) within the first TMD, were expressed in *Δyta10* and w303-1a (wt) and analyzed by Western blotting. Sequence of the first TMD of Mgm1p variants are presented above blots. Position of P, K, and D were numbered from the N-terminal of TMD. (PXnP, n= number of residues between two prolines)

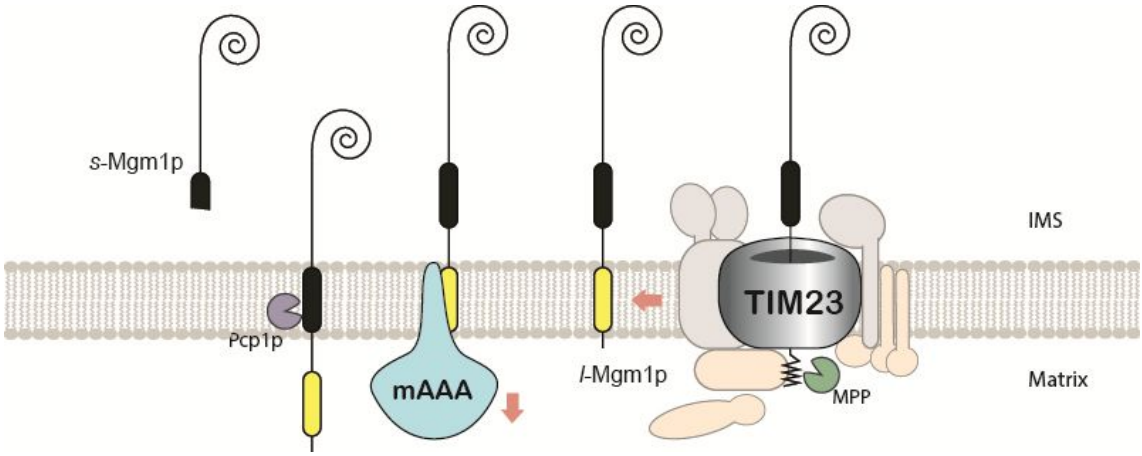


Fig. 13 Schematics of membrane protein dislocation by the m-AAA protease At the level of the TIM23 complex, Mgm1p variants with a proline within its first TMD or with charged residues flanking the TMD are inserted to the IM. Then, these proteins are recognized and dislocated by the m-AAA protease.

DISCUSSION

There are many cellular processes that involve the mitochondria, such as energy metabolism, signaling processes, and apoptosis. Thus, mitochondrial dysfunction causes serious harm to the cell, resulting in various diseases. Proper localization of proteins and successful removal of deleterious proteins in this organelle are prerequisites for functional mitochondria, therefore, understanding of how mitochondrial proteins are sorted into the right compartments and how protein qualities are controlled is of great importance.

The TIM23 complex is a central translocon in the mitochondrial IM that mediates both lateral release of proteins to the IM, and import to the matrix. In order to understand how the TIM23 complex interacts with other components and how it recognizes proteins to insert and to import, site-directed mutagenesis study with the core subunits of the TIM23 complex was conducted. We attempted to address which parts of Tim17p and Tim23p are important, therefore introduced mutations at TMDs of these subunits. Among the mutants that are generated in this study, mutations at the TMD2 of Tim23p showed growth defect at non-permissive temperature to varying degrees.

Mutation at both ends of the TMD2 of Tim23p induced a detrimental effect, exhibiting severe growth defect at 37℃ and accumulating precursor proteins that are targeted to the matrix or to the IM. Whilst, a mutation at the N-terminal of TMD2, *tim23* 149G:W did not interfere with the import of matrix-targeted proteins, they increased membrane insertion of substrates that do not harbor import-promoting sequence features. We speculate that replacement of 149G to W caused decrease in membrane potential, since the phenotype of tim23 149G:W was compromised when grown at respiring condition. Membrane potential of mitochondrial IM affects the biogenesis of mitochondria extensively, as translocation of mitochondrial proteins is vastly dependent on this. Moreover, it was suggested that membrane potential influences the interaction between subunits of the TIM23 complex [10] and leads to structural changes [25]. Thus, it is possible that reduced membrane potential in *tim23* 149G:W caused alteration in its complex formation, consequently influencing its function in protein sorting. However, it is still speculative and further studies are needed to elucidate molecular mechanisms of the TIM23-

mediated import and membrane insertion.

The m-AAA protease is involved in various cellular processes; proteolysis of misfolded proteins, processing of proteins, MrpL32p and Ccp1p, and dislocation of membrane proteins in yeast [14]. With Mgm1p variants, we observed that the m-AAA protease recognizes and dislocates proteins having prolines in the TMD or charged residues at the ends of the TMD, which were integrated to the membrane at the level of the TIM23 complex, whilst proteins with charges in the middle of the TMD were recognized and imported to the matrix by the TIM23 complex. This finding implies that proteins, with characters that make them unstable in the membrane but are not distinguishable by the TIM23 complex, are extracted with the help of the m-AAA complex.

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국문초록

TIM23 복합체는 미토콘드리아 단백질을 내막으로 삽입하거나 기질로 수송하는데 관여한다. 그러나 이 복합체가 어떤 메커니즘으로 단백질의 최종 위치를 인식하여 미토콘드리아의 다른 두 구획으로 전달하는지 아직 잘 연구되어 있지 않다. TIM23 복합체의 작용 기작을 이해하기 위해서, 이 복합체를 형성하고 있는 주요한 서브유닛인 Tim17p과 Tim23p의 특정 위치에 돌연변이를 유도하여 이 돌연변이가 TIM23 복합체의 기능에 어떠한 영향을 미치는지 관찰하였다. 연구 결과, Tim23p의 두 번째 transmembrane domain (TMD)이 중요한 역할을 수행한다는 것을 알 수 있었고, 특히 기질 근처에 위치한 부위가 막단백질의 삽입에 영향을 주는 것을 관찰하였다.

또한 m-AAA protease가 TIM23 복합체에 의해 내막에 삽입된 단백질들 중, 이 단백질 TMD의 어떤 특성을 인식하여 막으로부터 분리해 내는지 연구하였다. 다양한 서열을 가진 Mgm1p를 이용하여 실험한 결과, Mgm1p의 TMD에 proline이 포함되어 있거나 기질에 가까운 TMD 부분에 음전하를 띄는 아미노산이 있을 때, m-AAA가 이를 인식하여 미토콘드리아의 내막으로부터 분리해 낸다는 것을 관찰하였다. 이는 TMD의 helix 구조가 깨졌을 경우, 또는 미토콘드리아의 기질 근처에 음전하의 아미노산을 가져 막전위에 의해 TMD가 미토콘드리아 내막에 위치하는 것이 불안정할 경우, m-AAA protease가 인식하여 내막으로부터 분리해 낼 가능성을 시사한다.

이 연구를 통해 미토콘드리아의 내막 단백질을 형성하는 데 TIM23 복합체와 m-AAA protease가 협력하여 작용한다는 것을 알 수 있었다.

주요어: TIM23 complex, 미토콘드리아 단백질 수송, 미토콘드리아 단백질 막 삽입, Tim23p, Tim17p, m-AAA protease, 단백질 품질 제어