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The Mgr2 subunit of the TIM23 complex regulates membrane insertion of marginal stop-transfer signals in the mitochondrial inner membrane

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The TIM23 complex mediates membrane insertion of presequence-containing mitochondrial proteins *via* a stop-transfer mechanism. Stop-transfer signals consist of hydrophobic transmembrane segments and flanking charges. Mgr2 functions as a lateral gatekeeper of the TIM23 complex. However, it remains elusive which features of stop-transfer signals are discriminated by Mgr2. To determine the effects of Mgr2 on the TIM23-mediated stop-transfer pathway, we measured membrane insertion of model transmembrane segments of varied hydrophobicity and flanking charges in Mgr2-deletion or -overexpression yeast strains. We found that upon deletion of Mgr2, the threshold hydrophobicity for membrane insertion, as well as the requirement for matrix-facing positive charges, is reduced. These results imply that the Mgr2-mediated gatekeeper function is important for controlling membrane sorting of marginal stop-transfer signals.

Keywords: hydrophobicity; import; Mgm1; TIM23; yeast

The majority of integral membrane proteins have tightly packed α-helical transmembrane (TM) segments. The ability to form an α-helix and overall hydrophobicity are crucial determinants for the translocon-mediated recognition and insertion of a TM segment into the lipid bilayer, suggesting a direct interaction between the TM helix and the surrounding lipids during membrane insertion [1]. An earlier study has shown that the overall hydrophobicity required for TM helix insertion into the mitochondrial inner membrane (MIM) is comparable to what is required for TM helix insertion into the endoplasmic reticulum (ER) membrane [2]. Membrane sorting into the MIM is also affected by charged residues flanking a TM helix [3]. In spite of similar functions in protein translocation and membrane insertion, the Sec61 translocon in the ER and the TIM23 translocon complex in the MIM are evolutionarily unrelated [4,5]. Therefore, their structural architecture and modes of action may be different, but our current mechanistic understanding is rather limited.

The TIM23 complex is the main channel for import and membrane insertion of presequence-containing proteins into the MIM. It is composed of the membrane-spanning components Tim23, Tim17, Tim50, Tim21, and the recently found subunit Mgr2 [6]. Mgr2 was first identified as a subunit that mediates coupling of the TIM23 complex with the TOM

Abbreviations
ER, endoplasmic reticulum; IMS, intermembrane space; MIM, mitochondrial inner membrane; TM, transmembrane; WT, wild-type.

complex and respiratory chain complexes [6–8]. After translocation of the matrix-targeting presequence across the MIM, a downstream 'stop-transfer' signal halts translocation and is laterally released into the lipid bilayer [9,10]. It has been shown that membrane sorting of stop-transfer signals of the dynamin-related protein Mgm1 and cytochrome b_2 (Cyb2) precursors was enhanced in the absence of Mgr2, whereas the lateral release of these precursor proteins into the membrane was delayed upon Mgr2 overexpression [11]. A detailed site-specific crosslinking analysis of the interactions between the Cyb2 MIM sorting signal stalled at the TIM23 complex and Mgr2 demonstrated that Mgr2 makes contacts with both the hydrophobic segment and the matrix-facing positively charged amino acid residues. This result led to the proposal that Mgr2 functions as a lateral gatekeeper of the TIM23 complex by promoting recognition of stop-transfer signals and controlling their lateral release into the phospholipid bilayer [11]. However, the molecular basis of such a recognition process remains unknown.

To determine how Mgr2 regulates TIM23-mediated recognition of stop-transfer signals and TM helix insertion into the MIM, we measured membrane insertion of Mgm1 variants carrying a model TM helix of systematically varied hydrophobicity, and a set of Mgm1 mutants carrying substitutions of the flanking charged amino acid residues of TM1 [2,3]. We find that the threshold hydrophobicity for membrane insertion (50% membrane insertion) is reduced upon Mgr2 deletion. In particular, membrane insertion of moderately hydrophobic model TM segments or highly hydrophobic TM segments with mutations in charged flanking residues was notably increased in an Mgr2-deletion strain, indicating that Mgr2 is involved in regulating membrane-partitioning of marginal stop-transfer signals.

Materials and methods

Yeast strains and plasmids

The *Saccharomyces cerevisiae* wild-type (WT) YPH499 (*MATa*, *ade2, lys2, his3, leu2, trp1, ura3*), *MGR2*-deletion (*mgr2*Δ; YPH499, *mgr2::KANMX6*), and Mgr2-overexpression (Mgr2↑; *mgr2*Δ [pPGK-MGR2]) strains were used in this study [6,11–13]. The plasmids encoding Mgm1 variants in pHP84HA vector [2] were transformed into YPH499, *mgr2*Δ, and Mgr2↑ strains. Yeast transformants were cultured in –Leu (YPH499 and *mgr2*Δ) or –Leu –Ura (Mgr2↑) synthetic defined medium (0.67% Bacto yeast nitrogen base dropout amino acid mix, and 2% glucose or 3% glycerol, and 0.2% glucose) at 30 °C.

Protein preparation, SDS/PAGE, and western blotting

Yeast transformants expressing Mgm1 variants carrying mutated charged flanking residues were cultured in synthetic defined medium supplemented with 3% glycerol and 0.2% glucose, as used in Ref. [11], and Mgm1 variants carrying a set of L/A segment were cultured as in Ref. [2] at 30 °C overnight. Proteins were prepared as whole-cell lysates [2,14]. Whole-cell lysates were prepared from 10 OD_{600} units of cells grown to an OD_{600} of 0.6–0.8. Cells were lysed by addition of 1× lysis buffer (50 mM Tris/HCl pH 7.5, 5% SDS, 5% glycerol, 50 mM DTT, 5 mM EDTA, bromophenol blue, 2 $\mu g \cdot mL^{-1}$ Leupeptin, 2 $\mu g \cdot mL^{-1}$ Pepstatin A, 1 $\mu g \cdot mL^{-1}$ Chymostatin, 0.15 $mg \cdot mL^{-1}$ Benzamidine, 0.1 $mg \cdot mL^{-1}$ Pefabloc, 8.8 $\mu g \cdot mL^{-1}$ Aprotinin, 3 $\mu g \cdot mL^{-1}$ Antipain). Cell debris was removed by centrifugation at 20 000 ***g*** for 5 min. Cleared lysates were incubated at 55 °C for 15 min prior to SDS/PAGE. The samples were separated on 6.5% Tris/HCl gels (Bio-Rad, Hercules, CA, USA) for western blotting. Membranes were probed with an anti-HA antibody (Covance, Princeton, NJ, USA) and developed with Lumigen ECL Ultra kit on a Chemi-doc-XRS+ system (Bio-Rad). Quantification of detected bands was done using IMAGE LAB 5.0 (Bio-rad). Relative amounts of *l*-Mgm1 were quantified as [*l*-Mgm1/ (*l*-Mgm1 + *s*-Mgm1)] × 100(%).

Results and Discussion

Model protein

It has been shown that Mgr2 makes extensive contacts with both the hydrophobic region and the matrix-facing positively charged residues of a stop-transfer signal [11]. However, a systematic dissection of these features that Mgr2 helps to recognize prior to MIM sorting has not yet been conducted. To perform this analysis, we made use of the dual topology MIM protein Mgm1. Mgm1 exists naturally in two isoforms: *l*-Mgm1 and *s*-Mgm1 [15,16]. *l*-Mgm1 is generated when TM1 of Mgm1 is inserted into the MIM by the TIM23 complex (Fig. 1A, right). *s*-Mgm1 is generated when TM1—containing an inefficient stop-transfer signal—is translocated across the MIM and the downstream rhomboid cleavage site in TM2 is processed by Pcp1 in the MIM (Fig. 1A, left). Due to the unique characteristics of the TM1 of Mgm1, being at the threshold of membrane insertion by the TIM23 complex, Mgm1 has been useful to assess the effects of Mgr2 on the propensity of TM1 to undergo matrix translocation or MIM sorting [11].

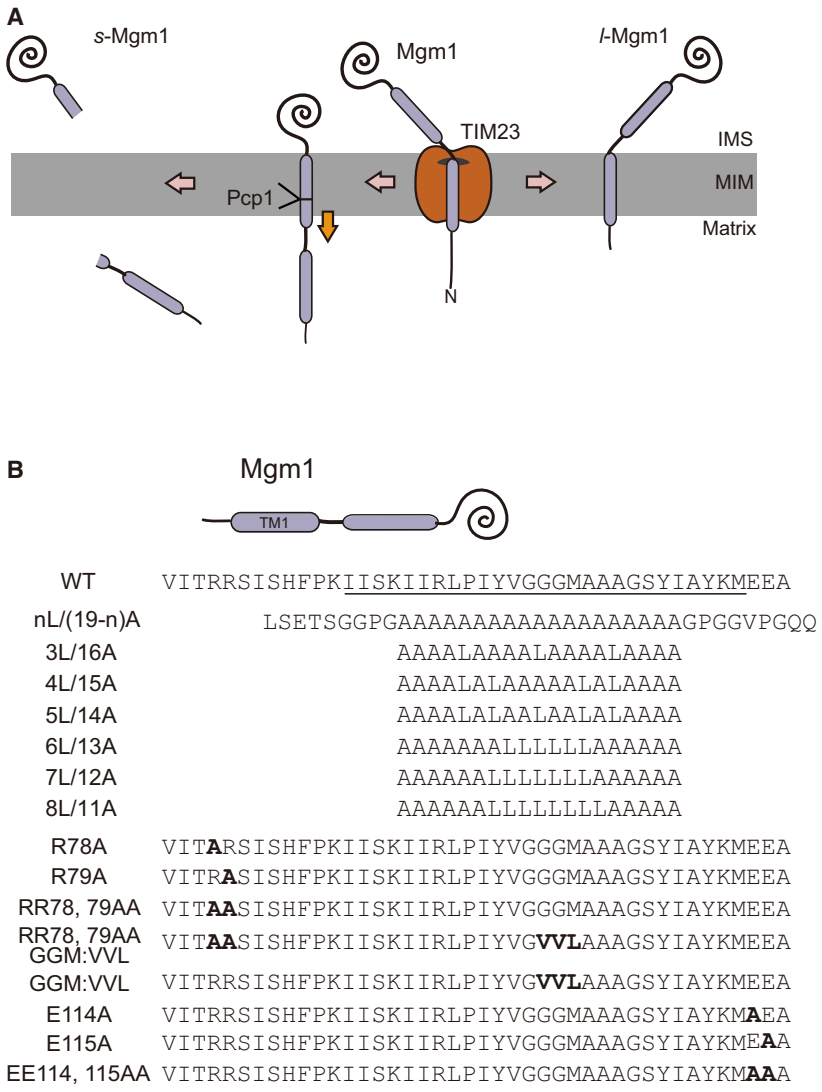

Fig. 1. Dual topology of Mgm1. (A) Schematics of topogenesis of Mgm1. When TM1 of Mgm1 is integrated into the MIM, the membrane-anchored long isoform, *l*-Mgm1 is generated (right). When TM1 is translocated into the matrix, TM2 is processed by Pcp1, cleaved into a short isoform, and *s*-Mgm1 is generated in the IMS (left). (B) Mgm1, sequences of and surrounding TM1 are shown. The underlined Mgm1 residues were replaced with nL/(19-*n*)A segments listed below. Mgm1 with mutated charged residues are indicated in bold.

The threshold hydrophobicity for TM helix insertion is reduced by deletion of Mgr2

When TM1 of Mgm1 was replaced by 19 amino acid stretches composed of *n* leucine and (19-*n*) alanine residues [Mgm1 (L/A) variants; Fig. 1B], the relative amounts of *l*-Mgm1 and *s*-Mgm1 that were produced correlated with the hydrophobicity of the TM1 segments as expected: the higher the hydrophobicity, the higher the fraction of *l*-Mgm1 (Fig. 2A,B) [2].

To systematically assess the effects of Mgr2 on TIM23-mediated TM helix recognition, we analyzed membrane insertion of a set of Mgm1 (L/A) variants in Mgr2-deletion (*mgr2*Δ), Mgr2-overexpression (Mgr2↑), and WT strains (Fig. 2A,B). Compared to the isogenic WT strain, membrane sorting of Mgm1 variants carrying a moderately hydrophobic segment (5L/14A or 6L/13A) was significantly enhanced in the absence of Mgr2. Membrane sorting of less hydrophobic (3L/16A and 4L/15A) or more hydrophobic (7L/12A and 8L/11A) segments were minimally or not affected by Mgr2 deletion. For the 5L/14A Mgm1 variant, reduced level of *l*-Mgm1 was detected upon Mgr2 overexpression, suggesting that membrane insertion of a TM in this range of hydrophobicity is particularly sensitive to Mgr2 levels.

While 50% membrane insertion (i.e., threshold hydrophobicity) was reached at $n = \sim 5–6$ leucines in the WT strain, the number of leucines required for threshold hydrophobicity was decreased to $n = \sim 4–5$ when Mgr2 was deleted and remained at ~ 5–6 L when Mgr2 was overexpressed (Fig. 2B).

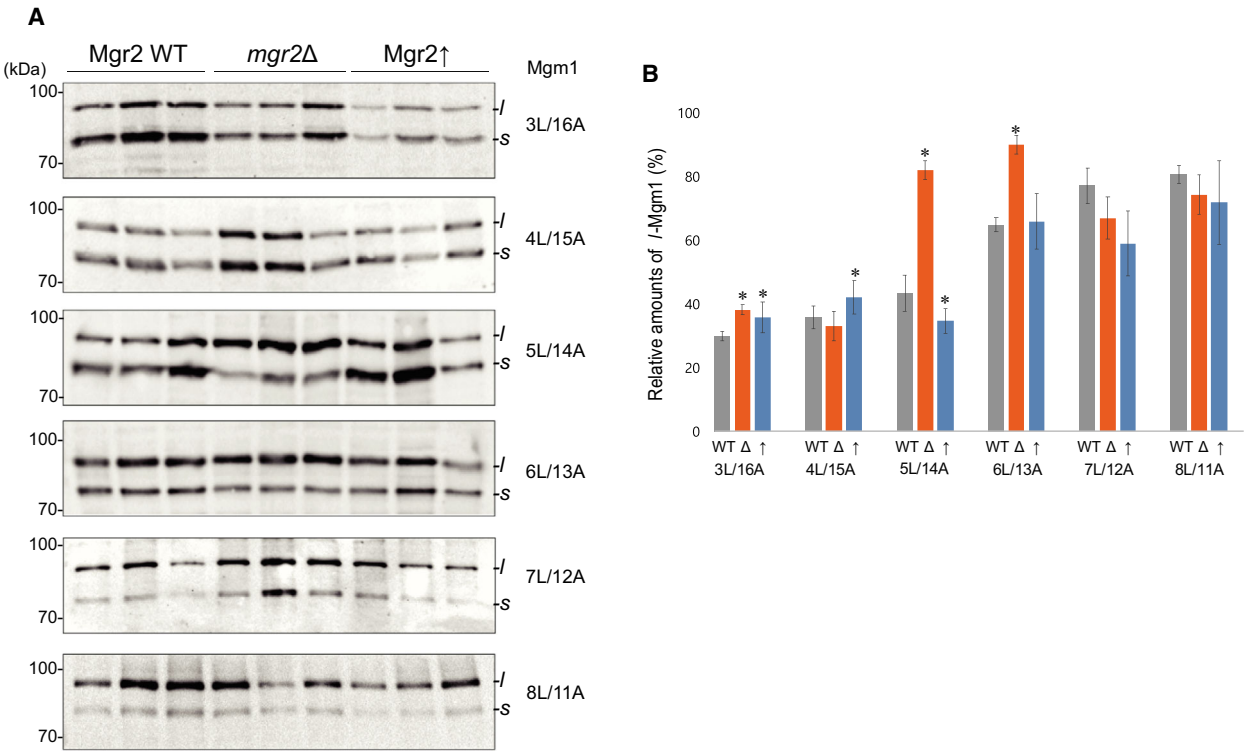

Fig. 2. Membrane insertion efficiency of Mgm1 (L/A) variants is modulated by Mgr2. (A) Whole-cell lysates of Mgr2-WT (WT), -deletion (Δ), or -overexpression (↑) cells expressing Mgm1 (L/A) constructs were analyzed by SDS/PAGE and western blotting with an α-HA antibody. *l*-Mgm1 (*l*) and *s*-Mgm1 (*s*) bands are labeled. Each construct was tested at least three times independently. (B) Relative amounts of *l*-Mgm1 of Mgm1 (L/A) variants in Mgr2-WT, -deletion (Δ), and -overexpression (↑) cells were quantified from (A) using IMAGE LAB (Bio-Rad) and plotted. A *t*-test was performed to check the statistical significance of the observed differences relative to the WT strain. * Marks samples with $P < 0.05$.

Effects of charged flanking residues on membrane insertion of Mgm1 in Mgr2-deletion and -overexpression strains

It has been shown that not only the hydrophobicity of a TM helix but also the flanking charged residues contribute to the stop-transfer function and the overall efficiency of membrane insertion by the TIM23 complex [2,3,17,18]. In the case of Mgm1, replacing the N-terminal positively charged residues flanking TM1 (Mgm1 R78A and R79A) significantly decreased membrane sorting, implying that the presence of these two arginine residues at the matrix side of TM1 is important for membrane sorting of this segment [3]. Notably, the positively charged segment of the MIM sorting signal of Cyb2 was shown to be efficiently crosslinked to Mgr2 [11]. Thus, it was hypothesized that Mgr2 may have a high affinity for the charged residues at the matrix-facing side of the TM and thereby control lateral insertion into the membrane.

We thus asked whether the charged residues flanking a TM segment are critical for the gatekeeper function of Mgr2. To this end, Mgm1 variants with substituted charged flanking residues (Fig. 1B) [3] were expressed in Mgr2-deletion (*mgr2*Δ), Mgr2-overexpression (Mgr2↑), and isogenic WT strains (Fig. 3A,B). As a control, we confirmed that for unmodified Mgm1 the fraction of membrane-inserted *l*-Mgm1 was increased relative to soluble *s*-Mgm1 in Mgr2-deletion (*mgr2*Δ), whereas decreased in Mgr2-overexpression (Mgr2↑) strain. (Fig. 3A,B) [3]. We found that membrane insertion was considerably reduced for Mgm1 R78A, Mgm1 R79A, and Mgm1 RR78, 79AA relative to unmodified Mgm1 in WT cells. The observed membrane sorting defects could be only marginally rescued (in the cases of Mgm1 R78A or RR78, 79AA) or not rescued (in the case of Mgm1 R79A) by the lack of Mgr2. We then used the Mgm1 RR78, 79AA variant made more hydrophobic by replacing three amino acids within TM1 (GGM to VVL mutation). The membrane insertion was found to be significantly increased in the *mgr2*Δ strain (Fig. 3). Because the presence or absence of Mgr2 affects the sorting behavior of the RR78,79AA variant in this context, we

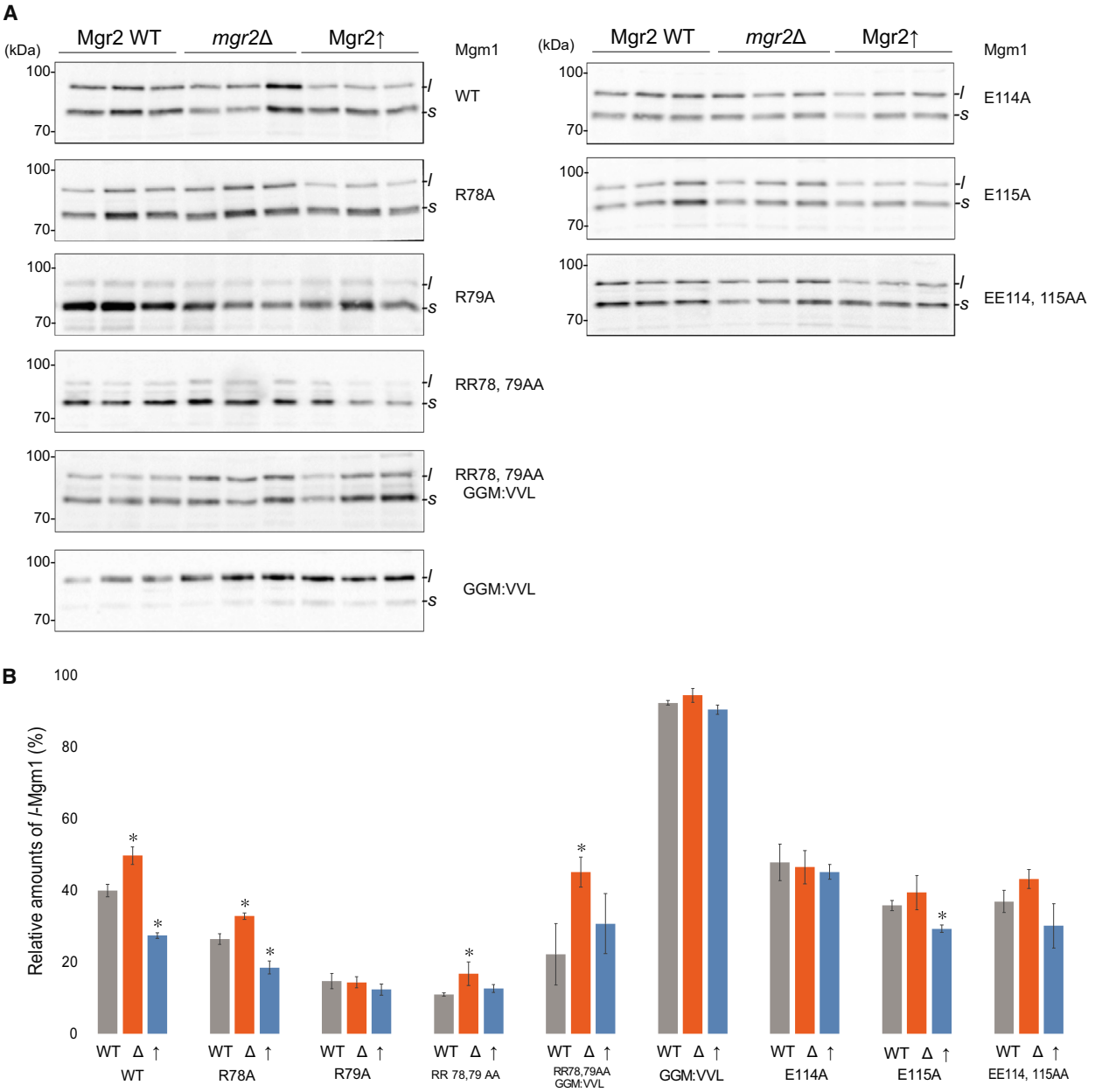

Fig. 3. Effects of Mgr2 on membrane insertion of Mgm1 *via* charged flanking residues. (A) Whole-cell lysates of Mgr2 WT, *mgr2*Δ, or Mgr2↑ cells expressing Mgm1 WT or variants with mutated flanking charged residues were analyzed as described in Fig. 2. (B) Relative amounts of *l*-Mgm1 of Mgm1 variants in Mgr2-WT, -deletion (*mgr2*Δ), and -overexpression (Mgr2↑) cells were quantified from (A) using Image Lab (Bio-Rad) and plotted. $P < 0.05$, denoted as *.

conclude that positive charges at the matrix-facing side of the TM segments cannot be critical for the recognition of Mgm1 TM1 by Mgr2. In comparison, membrane insertion of the more hydrophobic Mgm1 GGM:VVL variant without mutations in the charged flanking residues was unaffected by Mgr2. Next, membrane sorting of Mgm1 variants with mutations in negatively charged flanking residues at the intermembrane space (IMS)-facing C-terminal end of TM1 was assessed. The only difference we noticed was a mild decrease in membrane insertion for Mgm1 E115A in the Mgr2-overexpression strain (Fig. 3). Taken together, these results suggest that efficient stop-transfer signals are not considerably influenced by Mgr2, whereas 'moderate' stop-transfer signals that have reduced hydrophobicity or that have high

hydrophobicity but lack matrix-facing positively charged residues are subjected to the lateral gatekeeper function of Mgr2.

A role of Mgr2 in the sorting of TM segments has been proposed; however, the determining factors and their relative contribution to Mgr2-mediated regulation of membrane insertion were poorly defined. Our systematic quantitative assessment of MIM sorting of well-defined stop-transfer signals in a model preproteins sheds light on how Mgr2 acts on sorting of hydrophobic segments in the MIM.

Threshold hydrophobicity for the TIM23-mediated membrane insertion of Mgm1 variants is decreased in the absence of Mgr2, suggesting that hydrophobic partitioning into the MIM is facilitated in the absence of Mgr2. However, the membrane insertion of test hydrophobic segments was not linearly increased across the range of hydrophobicity, but the effects of Mgr2 were pronounced for the marginally hydrophobic TM segments. Consistent with this notion, Mgr2 was reported to influence membrane sorting of the TM segment of Cyb2 which is moderately hydrophobic [11,14,19]. We also found that in the case of highly hydrophobic segments, substitution of matrix-facing positively charged residues restores control of membrane sorting by Mgr2. We suggest that strong or weak stop-transfer signals are likely to be quickly equilibrated to the membrane and to the matrix, respectively, whereas moderate stop-transfer signals linger between the lipids and the protein channel relatively longer, sampling the membrane partitioning. A partial opening of the lateral gate in the absence of Mgr2 may have a major impact in membrane sorting of marginal stop-transfer signals.

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Author contributions

GvH and HK conceived and supervised the study. SL, HL, GvH and HK designed experiments. SL, HL, and SY performed experiments. RI and MvdL provided new reagents. SL, HL, SY, RI, MvdL, GvH and HK analysed data. SL, HL, RI, MvdL, GvH and HK wrote the manuscript. SL, RI, MvdL and HK made manuscript revisions.

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