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RESEARCH ARTICLE

The Sec62–Sec63 translocon facilitates translocation of the C-terminus of membrane proteins

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ABSTRACT

The Sec62–Sec63 complex mediates post-translational translocation of a subset of primarily secretory proteins into the endoplasmic reticulum (ER) in yeast. Therefore, it has been thought that membrane proteins, which are mainly co-translationally targeted into the ER, are not handled by the Sec62–Sec63 translocon. By systematic analysis of single and multi-spanning membrane proteins with broad sequence context [with differing hydrophobicity, flanking charged residues and orientation of transmembrane (TM) segments], we show that mutations in the N-terminal cytosolic domain of yeast Sec62 impair its interaction with Sec63 and lead to defects in membrane insertion and translocation of the C-terminus of membrane proteins. These results suggest that there is an unappreciated function of the Sec62–Sec63 translocon in regulating topogenesis of membrane proteins in the eukaryotic cell.

KEY WORDS: Endoplasmic reticulum, ER, Co-translational translocation, Sec61, Topology, Yeast

INTRODUCTION

Secretory and membrane proteins destined for the subcellular organelles or the plasma membrane are first targeted to the endoplasmic reticulum (ER) by a co- or post-translational translocation pathway (Rapoport, 2007). In the co-translational translocation pathway, as a nascent chain emerges from the ribosome and a signal recognition particle (SRP) recognizes a stretch of hydrophobic residues in the N-terminus of the nascent chain, called a signal sequence. Then, the SRP targets the nascent chain to the Sec61 translocon, the main translocation machinery in the ER membrane. This translocon is a heterotrimeric complex composed of Sec61p/Sec61α, Sbh1p/Sec61β and Sss1p/Sec61γ (the first name is that in yeast and the second its human homolog), where Sec61p/Sec61α is the pore-forming subunit (van den Berg et al., 2004; Rapoport, 2007). The majority of proteins that are co-translationally translocated into the ER are membrane proteins as this prevents premature aggregation of hydrophobic domains in the cytosol.

Many secretory proteins, however, are targeted post-translationally, and cytosolic chaperons are involved in keeping the polypeptide chains in an unfolded, translocatable state (Chirico et al., 1988; Deshaies et al., 1988; Zimmermann et al., 1988; Plath and Rapoport, 2000). In yeast, post-translational translocation requires, in addition to the trimeric Sec61 complex, the Sec62–Sec63 complex, consisting of Sec62p, Sec63p, Sec71p and Sec72p subunits (Panzner et al., 1995; Lyman and Schekman, 1997; Harada et al., 2011). Sec62p and Sec63p are essential proteins in yeast and are also found in all eukaryotes (Rothblatt et al., 1989; Meyer et al., 2000; Tyedmers et al., 2000; Müller et al., 2010). The 30-kDa Sec62p is an integral membrane protein with two transmembrane (TM) domains, and both the N- and C-terminus are located in the cytosol (Deshaies and Schekman, 1989; Deshaies and Schekman, 1990). The N-terminal cytosolic domain is rich in positively charged residues and has been shown to interact with the negatively charged C-terminal domain of Sec63p (Wittke et al., 2000; Willer et al., 2003).

Neither the mechanism of post-translational translocation nor the mode of action of the Sec62–Sec63 complex is well understood. In the post-translational translocation process, it has been proposed that the Sec62–Sec63 complex might recognize the signal sequences of proteins (Ng et al., 1996). However, Sec63p is essential for both co- and post-translational translocation (Brodsky et al., 1995; Young et al., 2001), and the two non-essential components of the Sec62–Sec63 complex, Sec71p and Sec72p were first discovered from genetic screening for proteins that could rescue membrane protein translocation defects (Green et al., 1992). Furthermore, the mammalian Sec62 has been found to contain a ribosome-binding domain, implying that it could be involved in co-translational translocation (Meyer et al., 2000; Müller et al., 2010). Recently, we have shown that Sec62 mediates insertion of moderately hydrophobic signal anchor proteins into the membrane at an early stage of translocation (Reithinger et al., 2013). Therefore, these findings imply that the function of the Sec62–Sec63 complex might not be limited to the post-translational translocation pathway.

To further characterize the role of Sec62 and to assess whether it has a more general role in the biogenesis of membrane proteins, we prepared a set of Sec62 mutants and tested the effects of the mutations on translocation and membrane insertion of engineered single- and multi-spanning membrane proteins in the yeast *Saccharomyces cerevisiae*. Our results show that mutations in the cytosolic, N-terminal domain of Sec62 disrupt the interaction between Sec62 and Sec63, and impair translocation of the C-terminus of membrane proteins, suggesting that the Sec62–Sec63 complex is involved in topogenesis of membrane proteins in the ER.

RESULTS

Selection of viable Sec62 mutants

Sec62 is a double-spanning membrane protein with its large N- and C-terminal soluble domains in the cytosol (Fig. 1A). To investigate the function of Sec62, we prepared a set of Sec62 mutants with mutations in the transmembrane, N-terminal or the C-terminal domains of Sec62 (Fig. 1B). The bulky hydrophobic

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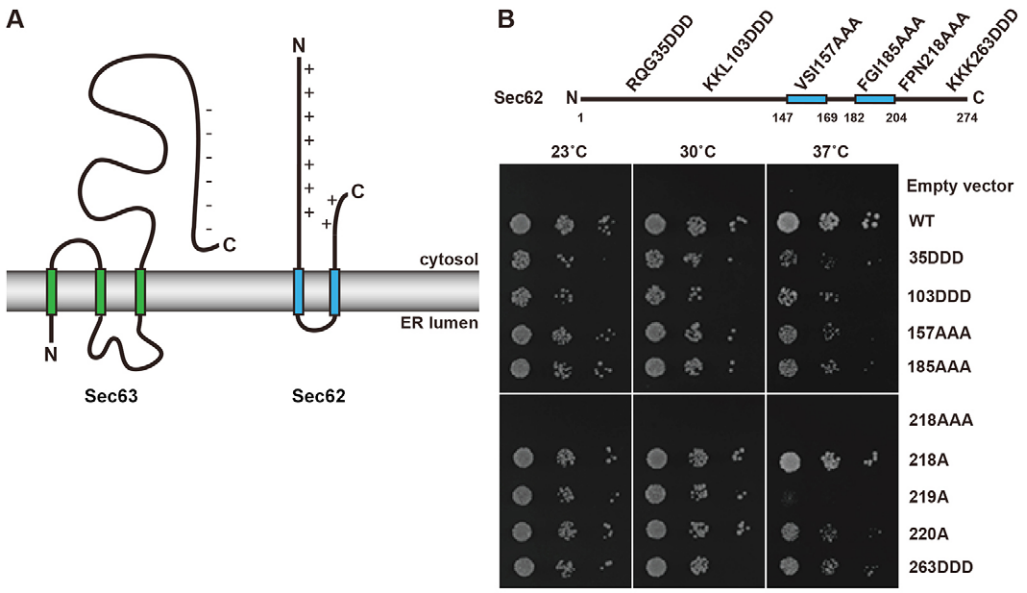

Fig. 1. Viability of Sec62 mutant strains. (A) Schematic representation of Sec63 and Sec62. The charged domains, (+) and (−), are schematically indicated. (B) Positions of mutations in Sec62 and viability of Sec62 mutant strains. Mutated residues are indicated with the sequences and the starting residue number, followed by the mutated sequences. JRY4 strains were transformed with a plasmid carrying *SEC62* WT or the indicated *sec62* mutants. The cells were grown on 5-fluoroorotic acid (FOA) agar plate for 3 days at 23˚C, 30˚C and 37˚C. OD_{600}=0.600/ml cells (~3×10^7 cells) were diluted (1:100) and 10 µl was used for the first spot. The following spots were 10-fold serially diluted.

residues in TM1 (157–159, VSI) and in TM2 (185–187, FGI) were replaced with Ala residues (157AAA and 185AAA). A cluster of positively charged residues in the N-terminal domain has previously been shown to be important for the formation of the Sec62–Sec63 complex (Wittke et al., 2000; Willer et al., 2003); thus, we replaced the positively charged residues at positions 35–37 (RQG to DDD; 35DDD) or at 103–105 (KKL to DDD; 103DDD) with negatively charged residues. We chose residues 35–37, because a *sec62-1* mutant strain with residue 37 mutated to Asp has been shown to impair post-translational translocation (Wittke et al., 2000). A cluster of positively charged residues are also found in the C-terminal domain, but the functional significance of this domain is so far unknown. We replaced Lys residues at positions 263–265 in the C-terminal domain with Asp (263DDD). In addition, the C-terminal cytosolic domain following TM2 has been shown to be essential for cell viability (Wittke et al., 2000), thus residues 218–220 (FPN) in this domain were replaced by Ala (218AAA). All Sec62 variants were transformed into JRY4, a *sec62* deletion strain, containing a Ura-bearing plasmid encoding the wild-type (WT) *SEC62*. The ability to complement *sec62* deletion was tested by plasmid shuffling (Fig. 1B). We found that all Sec62 variants, except 218AAA, could functionally replace the WT (Fig. 1B). Although P219A showed a growth defect at 37˚C, the single mutants F218A, P219A and N220A, complemented the *sec62* deletion.

Translocation and membrane insertion assay

To determine the changes in translocation and insertion of membrane proteins into the ER membrane in the various *sec62* mutant strains, we carried out a translocation and membrane insertion assay with a series of model membrane proteins containing one, two or three TM domains. These model proteins were derived from *E. coli* leader peptidase (LepB). Efficient N-glycosylation sites were engineered at various positions in the model proteins that enabled us to distinguish between model proteins with more than one orientation. Furthermore, the hydrophobicity of the TM domains was fine-tuned by varying the number of Leu (hydrophobic) and Ala (neutral) residues to select for marginally hydrophobic TM domains that are most sensitive in probing a successful translocation, non-insertion or membrane insertion. These model proteins have been successfully used in yeast and/or *in vitro* mammalian microsome systems (Lundin et al., 2008; Öjemalm et al., 2012; Reithinger et al., 2013).

Translocation and membrane insertion assay with signal anchor proteins

We first tested a set of model membrane proteins with one TM domain (H1 model protein) in our various *sec62* mutants (Fig. 2A). As we recently reported that the *sec62-1* mutant impairs targeting and membrane insertion of moderately hydrophobic signal anchor proteins (Reithinger et al., 2013), we wanted to see whether mutations in other regions of Sec62 also had the same effect.

H1 model proteins were made by replacing the two N-terminal TM domains of Lep with a hydrophobic segment (H-segment) carrying Ala and Leu residues. This H-segment functions both as a signal sequence and a TM domain (Fig. 2A). The H1 model proteins have an asymmetric distribution of the glycosylation sites on either side of the TM domain that allows us to assess not only ER targeting but also membrane topology of the model protein: an unglycosylated protein (0G) indicates a non-targeted protein, a singly glycosylated protein (1G) indicates that the protein inserted into the membrane in an N_{out}-C_{in} orientation (i.e. the N-terminus is in the ER lumen, the C-terminus is in the cytosol), a doubly glycosylated protein (2G) indicates that the protein inserted in an N_{in}-C_{out} orientation (i.e. the N-terminus is in the cytosol, the C-terminus is in the ER lumen), and a triply glycosylated protein (3G) is a completely translocated protein. Model proteins with the H-segment compositions of four Leu and

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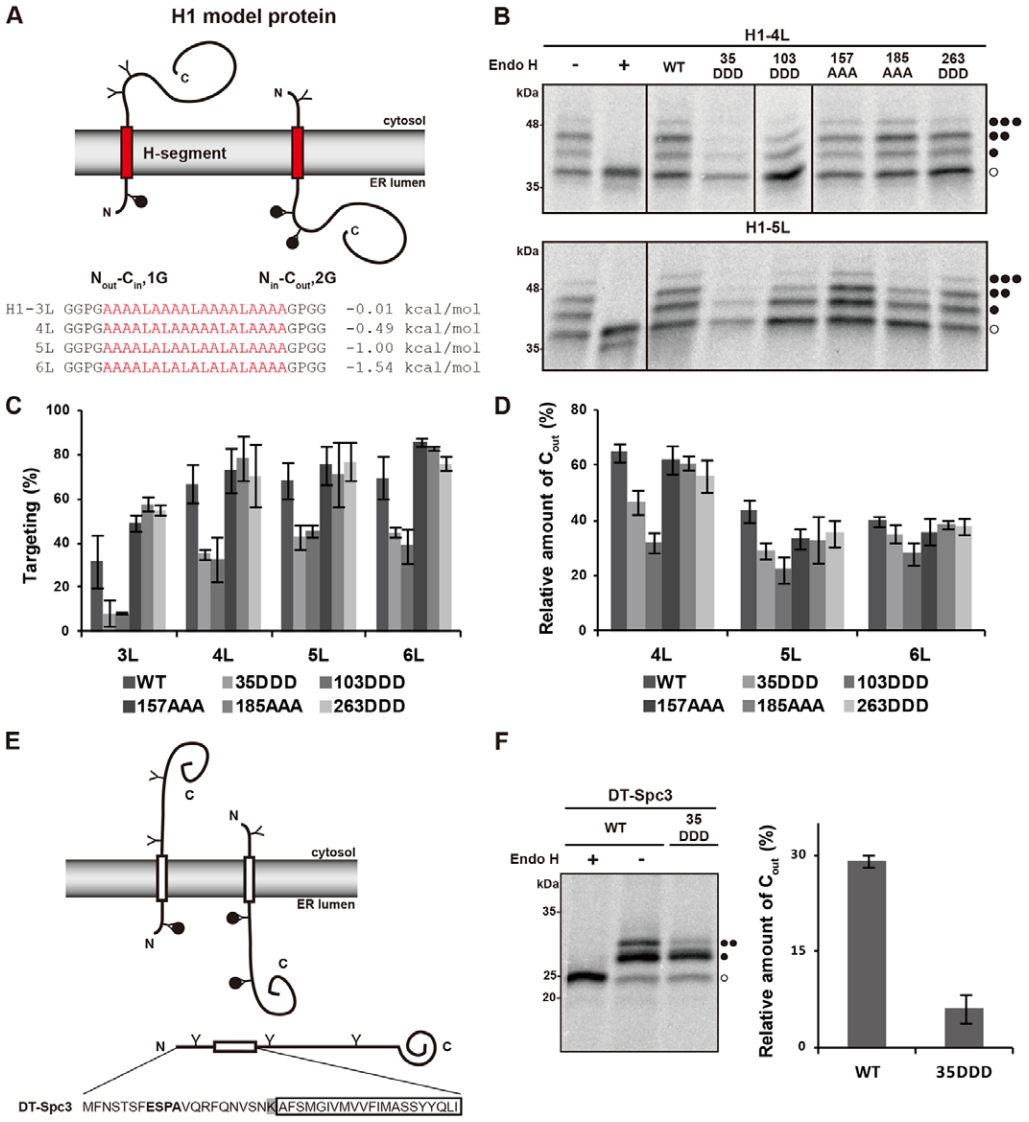

Fig. 2. Membrane insertion of single-spanning membrane proteins with a N_{in}-C_{out} orientation is reduced in *sec62* N-terminal mutant strains. (A) An *E.coli* Lep-based H1 model protein has a single potential TM segment (red) with varying numbers of leucine (L) and alanine (A) residues. It is equipped with one N-terminal and two C-terminal glycosylation sites, indicated as Y. Filled circles indicate modified glycosylation sites. The sequences with their apparent free energy of insertion (ΔG values in kcal/mol were predicted with a ΔG predictor) of the H1 model proteins carrying the H-segment compositions of 3L to 6L are shown. (B) H1-4L and H1-5L constructs were expressed in *SEC62* WT or the indicated mutant strains. Proteins were radiolabeled with [S^{35}]Met for 5 min at 30˚C and immunoprecipitated using anti-HA antibody. Samples were analyzed by SDS-PAGE and visualized by autoradiography. H1-4L and H1-5L expressed in *SEC62* WT were treated with or without EndoH to detect the unglycosylated form. Triply (3G), doubly (2G) and singly (1G) glycosylated products are indicated as three, two and one filled circles, respectively, and an unglycosylated product is indicated as an open circle. (C) Translocation efficiency of H1 model proteins as determined both by autoradiography and western blotting analysis was calculated as [(1G+2G+3G)/total×100]. Results are mean±s.e.m. (n=3). (D) The relative amount of N_{in}-C_{out} form of the same set of data in Fig. 2C was calculated as [2G/(1G+2G)×100]. Results are mean±s.e.m. (n=3). (E) Schematic representation of DT-Spc3. The signal anchor sequence is indicated (box) and the added or mutated residues are shown in bold or shaded. DT-Spc3 contains a C-terminal 3×HA tag. (F) DT-Spc3 was expressed in *SEC62* WT or *sec62* 35DDD strain and visualized by autoradiography as in B, except cells were radiolabeled at 25˚C. A WT sample was treated with or without Endo H, and glycosylated products are indicated as filled circles (right). The relative amount of N_{in}-C_{out} form was calculated as [2G/(1G+2G)×100]. Results are mean±s.e.m. (n=3).

five Leu (H1-4L and H1-5L) were expressed in *sec62* mutant strains, and detected either by western blotting with whole-cell lysates, or immunoprecipitation and autoradiography after pulse-labelling with [S^{35}]Met (Fig. 2B–D). To identify the unglycosylated (0G) band, samples were treated with endoglycosidase H (EndoH), as shown in the example in Fig. 2B.

When H1-4L and H1-5L were expressed in the 35DDD and 103DDD mutant strains, the amount of glycosylated products decreased compared to the WT, indicating a defect in targeting (Fig. 2B,C). Other *sec62* mutants retained WT levels of translocation (Fig. 2B,C). Furthermore, 35DDD and 103DDD *sec62* mutants affected topogenesis of H1 model proteins. Compared to the WT, the relative amount of proteins in N_{in}-C_{out} membrane topology was reduced in 35DDD and 103DDD *sec62* mutants by 18% and 32% for H1-4L, and 14% and 21% for H1-5L, respectively (Fig. 2D). Another signal anchor protein, signal peptidase complex 3 (Spc3), was also tested in WT and 35DDD strains. Spc3 was modified to insert with dual topology

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(DT-Spc3) (Reithinger et al., 2013). Similar to the H1 model protein, one and two glycosylation sites flank the TM domain of DT-Spc3 asymmetrically (Fig. 2E). In agreement with the H1 model protein data, when expressed in the 35DDD *sec62* mutant, the ratio of DT-Spc3 inserting in the N_{in}-C_{out} membrane topology decreased by about fivefold compared to the WT (Fig. 2F).

These results indicate that the mutations introduced in the N-terminal cytosolic domain of Sec62 reduce the efficiency of translocation of moderately hydrophobic signal anchor proteins as well as membrane insertion in the N_{in}-C_{out} orientation, consistent with our earlier findings with the *sec62-1* mutant (Reithinger et al., 2013).

Basic residues in the N-terminus of Sec62 are indispensable

To distinguish whether the introduction of negatively charged amino acids or the deletion of positively charged residues in Sec62 is responsible for the observed defect above, residues at positions 35–37 were replaced with neutral Ala residues. Then, H1 signal anchor proteins (H1-4L and H1-5L) were expressed in the 35AAA *sec62* mutant strain (Fig. 3A). Membrane insertion of H1 proteins into the ER membrane was decreased to a similar level to that seen in the 35DDD and 103DDD mutant strains (Fig. 3B,C), suggesting that positively charged residues in the N-terminus of Sec62 are indispensable.

Furthermore, to check whether the defects caused by the 35DDD and 103DDD mutations in Sec62 were additive, double mutant strains were constructed (35DDD+103DDD, 35DDD+263DDD and 103DDD+263DDD). Then, H1-4L and H1-5L were expressed in these *sec62* double mutant strains (Fig. 3A). Because the 263DDD mutant strain did not show any targeting defect for H1 model proteins, if the defects in 35DDD and 103DDD double mutants are not additive, all the other double mutants would be expected to exhibit a similar level of defect to that of the 35DDD or 103DDD single mutations. In all cases, insertion efficiency was similar to that of the single mutant strains (Fig. 3B,C). These results suggest that positively charged residues in the N-terminus of Sec62 are crucial for the folding of the N-terminal cytosolic domain and/or proper interaction with Sec63p.

Mutations in the N-terminus of Sec62 disrupt interaction with Sec63

Previous studies have indicated that an electrostatic interaction between the N-terminus of Sec62 and the C-terminus of Sec63 is important for formation of the Sec62–Sec63 complex (Wittke et al., 2000; Willer et al., 2003). To investigate whether the substitutions in this region of Sec62 disrupt the interaction between Sec62 and Sec63, a co-immunoprecipitation assay was carried out. FLAG-tagged Sec62 WT, 35DDD, 35AAA, 103DDD and 263DDD were expressed in cells containing HA-tagged Sec63. Crude membranes were isolated, solubilized with 1% Triton X-100 and subjected to immunoprecipitation with anti-HA antibodies. Both WT and 263DDD were co-precipitated, indicating a stable complex formation with Sec63 (Fig. 4). 35DDD, 35AAA and 103DDD, however, were not detected in immunoprecipitated samples (Fig. 4). 103DDD migrated on SDS-PAGE slightly slower than others (Fig. 4). These results suggest that replacement of positively charged residues in the cytosolic, N-terminal domain of Sec62 abolishes its proper interaction with Sec63.

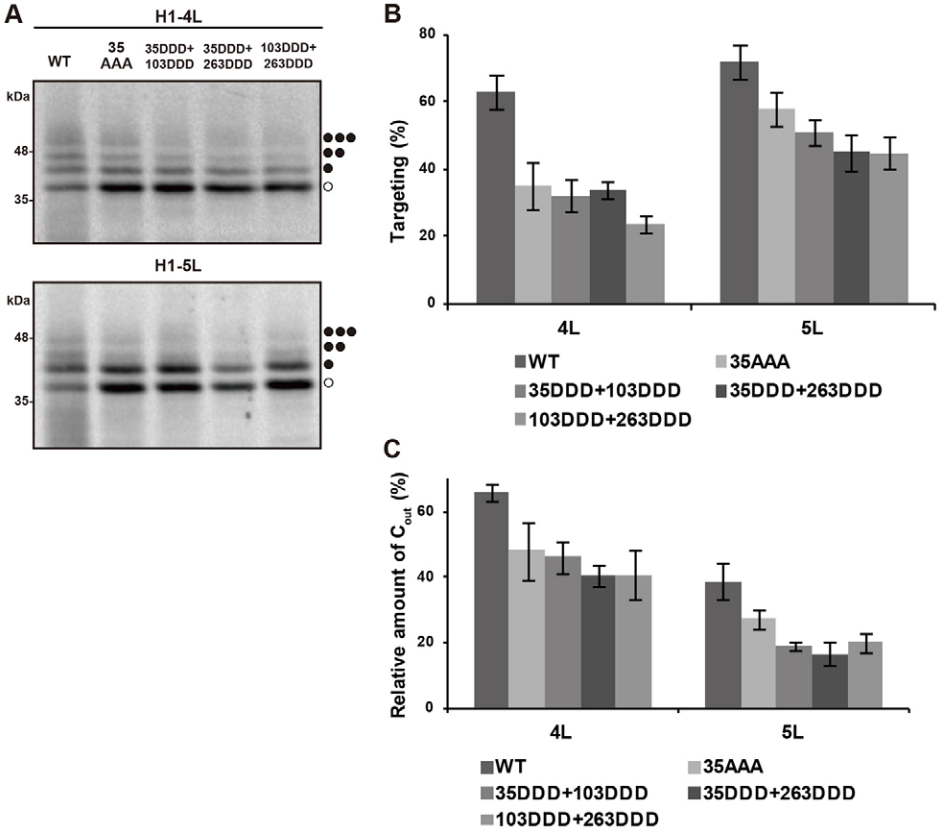

Fig. 3. Translocation assay with H1 model signal anchor proteins in Sec62 variants. (A) H1-4L and H1-5L expressed in Sec62 mutant strains were visualized by western blotting. Triply, doubly and singly glycosylated products are indicated as three, two and one filled circles, respectively, and an unglycosylated product is indicated as an open circle. (B) Translocation efficiency was calculated as [(1G+2G+3G)/total×100] and plotted. Results are mean±s.e.m. (n=3). (C) The relative amount of N_{in}-C_{out} orientation of H1 model proteins was calculated as [2G/(1G+2G)×100] and plotted. Results are mean±s.e.m. (n=3).

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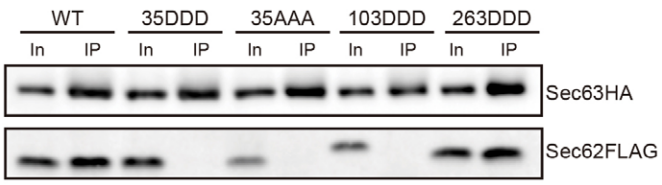

Fig. 4. Mutations in the N-terminal domain of Sec62 disrupt the interaction with Sec63. Crude membranes were solubilized with 1% Triton X-100 and immunoprecipitated (IP) with an anti-HA antibody (mouse) against Sec63 conjugated to an HA epitope. Samples were subjected to SDS-PAGE followed by western blotting with either anti-HA (rabbit, Sec63HA) or anti-FLAG (rabbit, Sec62FLAG) antibody. In, Input.

35DDD and 103DDD mutants inhibit insertion of the moderately hydrophobic second TM domain

To determine whether the defective Sec62–Sec63 complex affects membrane insertion of not only single-spanning membrane proteins, but also multi-spanning membrane proteins, we expressed an H2 model protein that has two TM domains in Sec62 mutant strains. This H2 model protein contains the original TM segment of *E. coli* Lep as the upstream TM domain, which targets the protein efficiently to the ER, and an H-segment comprised of varying numbers of Leu and Ala residues as the downstream TM domain (H2 segment) (Lundin et al., 2008) (Fig. 5A). Two glycosylation sites flanking the TM domains allow assessment of protein targeting and membrane insertion of the H2 model protein.

The H2 model proteins with H2 segment compositions of 0L (P2), 2L, 3L and 5L (Fig. 5A) were expressed in *SEC62* WT and

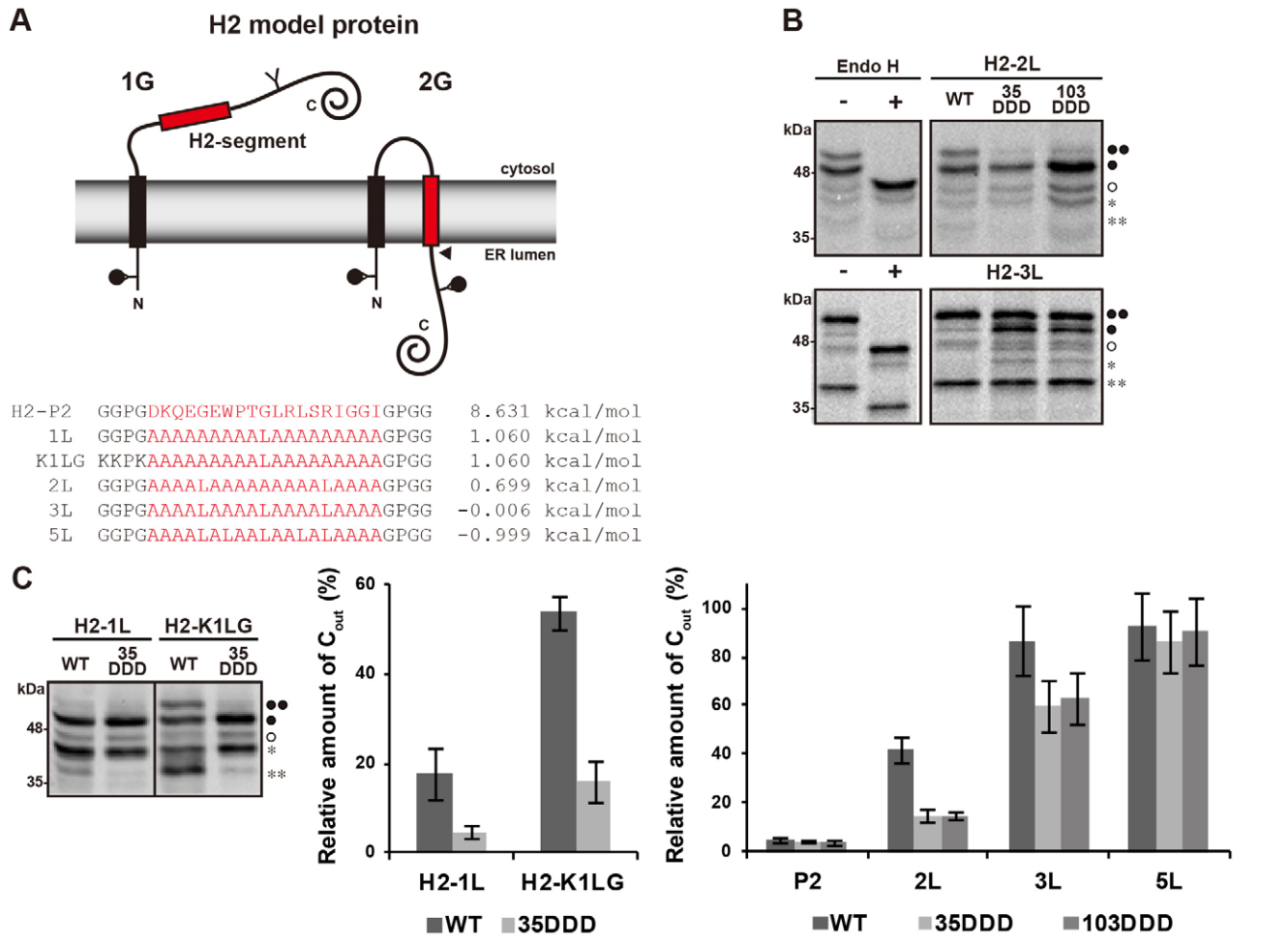

Fig. 5. Membrane insertion of double-spanning membrane proteins in Sec62 mutant strains. (A) *E.coli* Lep-based H2 model protein. The potential second TM segment (H2 segment) is indicated in red. Hydrophobicity of the H2 segment was controlled by changing the number of leucine (L) and alanine (A) residues. H2 model protein is equipped with N- and C-terminal glycosylation sites, which are indicated as Y. Filled circles indicate glycosylated glycosylation sites. The H2 model protein contains a C-terminal 3×HA epitope tag. The sequences with their apparent free energy of insertion (ΔG values in kcal/mol were predicted with ΔG predictor) of the H2 segment are shown. (B) H2-2L and H2-3L constructs were expressed in *SEC62* WT or mutant strains. Samples were analyzed as in Fig. 2B. Doubly (2G) and singly (1G) glycosylated products are indicated as two and one filled circles, respectively, and an unglycosylated product is indicated as an open circle; *, an unglycosylated C-terminal domain that resulted from an unknown cleavage and is detected only when the H2 segment is not membrane inserted. **, a glycosylated C-terminal domain that resulted from signal peptidase cleavage (arrowhead in A) (Lundin et al., 2008). WT samples were treated with or without Endo H. Membrane insertion efficiency of the H2 segment was calculated as [(2G+2G cleaved product)/(1G+1G cleaved product+2G+2G cleaved product)×100]. Results are mean±s.e.m. (n=5). (C) H2-1L and H2-K1LG constructs were expressed in *SEC62* WT or *sec62* 35DDD mutant strains. Whole-cell lysates were analyzed by SDS-PAGE and western blotting. Membrane insertion efficiency was calculated as in Fig. 4B. Results are mean±s.e.m. (n=4).

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mutant strains. In all strains, no or very little unglycosylated products were detected, indicating efficient ER targeting (Fig. 5B). Membrane insertion and, consequently, translocation of the C-terminus of the H2 segment of H2-2L, however, was more than twofold reduced in 35DDD and 103DDD strains (Fig. 5B). In addition, H2-3L also showed a small reduction in membrane insertion and translocation of the C-terminus in *sec62* 35DDD and 103DDD mutants (27% and 23%, respectively) (Fig. 5B). The more hydrophobic H2 segment, H2-5L was not affected (data not shown). Compared to H1 model proteins where the threshold hydrophobicity (50% membrane insertion) is ~4L–5L, H2 model proteins require less Leu residues for 50% insertion (~2L–3L), and this difference is likely to be due to the presence of the first TM segment (Lundin et al., 2008). Even though absolute hydrophobicity might differ depending on the context of the sequence (e.g. the presence of the upstream TM domain and/or the loop length), results from the two model proteins show that a defective Sec62–Sec63 complex impairs translocation of the C-terminus and membrane insertion of marginally hydrophobic TM segments.

Next, we investigated membrane insertion of a TM domain with charged flanking residues in Sec62 defective strains. Positively charged residues flanking the TM domain influence membrane orientation of the TM segments (Öjemalm et al., 2012). We prepared an H2 model protein with the H2 segment that was composed of 1 Leu and 18 Ala residues that were N-terminally flanked by three Lys residues (H2-K1LG, Fig. 5A). In WT cells, 54% of H2-K1LG was doubly glycosylated, whereas in 35DDD strain, only 16% were doubly glycosylated, indicating a defect in membrane insertion and translocation of the C-terminus (Fig. 5C). These data show that the defective Sec62–Sec63 complex impairs membrane insertion of not only a moderately hydrophobic TM domain but also a TM domain with charged flanking residues.

The C_{out} orientation is reduced in Sec62 mutant strains

Translocation of the C-terminus and membrane insertion of the H2 segment in the H2 model protein were impaired in Sec62 mutant strains. To distinguish whether the translocation of the C-terminal end or membrane insertion is defective in Sec62 mutant strains, we tested another model protein carrying three TM segments, with the C-terminus facing the cytosol when the last TM segment is membrane inserted (Fig. 6A). This model protein is derived from Lep IV (Öjemalm et al., 2012), which contains an original first TM domain of *E. coli* Lep, a hydrophobic second TM domain with 7 Leu and 12 Ala residues, followed by a potential third TM segment (H3 segment) of differing hydrophobicity. The first two TM segments were inserted efficiently into the ER membrane in WT and *sec62* mutants, as very little unglycosylated and singly glycosylated products were detected (Fig. 6B). In the *SEC62* WT strain, H3-4L and H3-5L model proteins show two distinct forms; the H3 segment was either membrane-inserted (2G) or translocated (3G). The relative amount of 3G form decreased in the 35DDD and 103DDD *sec62* mutant strains compared to the WT; there was a 28% and 24% decrease for H3-4L, and 22% and 13% for H3-5L in 35DDD and 103DDD mutants, respectively. These results indicate that the C_{out} membrane orientation compared to the C_{in} orientation was reduced in *sec62* mutant strains. It is of note that this effect was only observed for 4L and 5L constructs but not for less

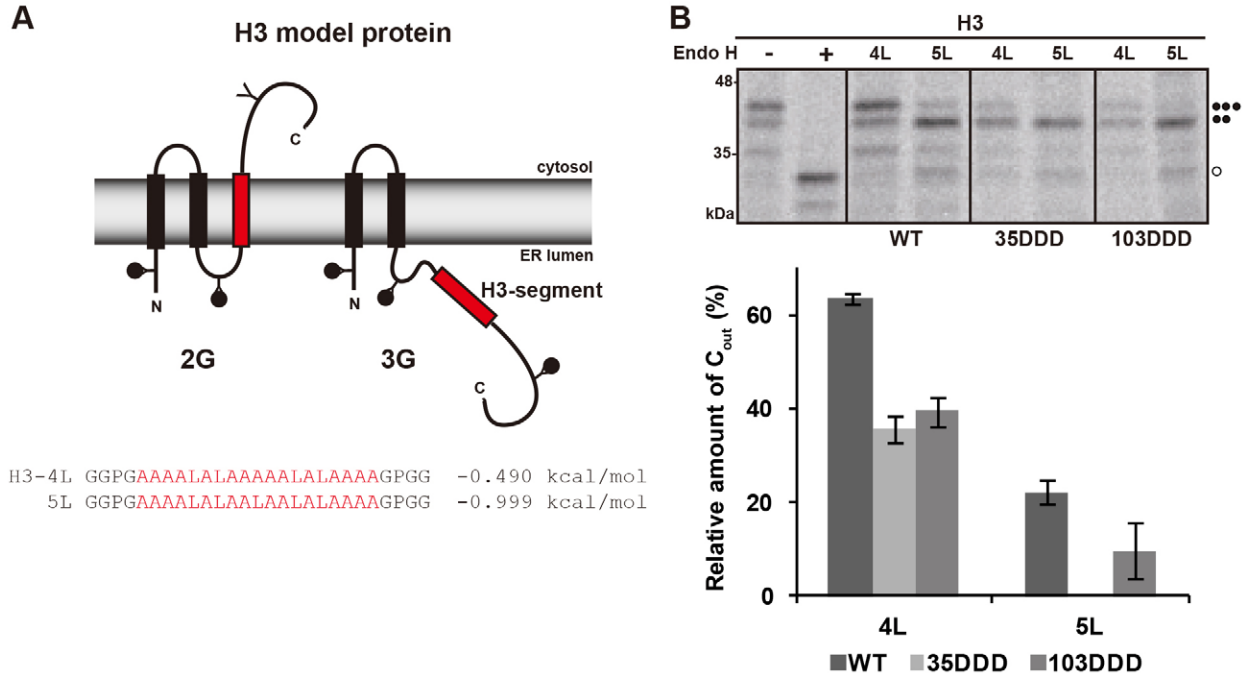

Fig. 6. Membrane insertion of multi-spanning membrane proteins in Sec62 mutant strains. (A) *E. coli* Lep-based H3 model protein. The potential third TM segment (H3 segment) is indicated in red and the sequences with their apparent free energy of insertion (ΔG values in kcal/mol were predicted with ΔG predictor) are shown. The H3 model protein is equipped with N- and C-terminal glycosylation sites which are indicated as Y, and an HA-tag at the C-terminus. Filled circles indicate glycans. (B) H3-4L and H3-5L constructs were expressed in *SEC62* WT or the indicated mutant strains. Samples were analyzed as in Fig. 2B. Triply (G) and doubly (2G) glycosylated products are indicated as three and two filled circles, respectively, and an unglycosylated product is indicated as an open circle. H3-4L expressed in Sec62 WT was treated with or without Endo H to detect the unglycosylated form. Efficiency of the C-terminal translocation was calculated as [3G/(2G + 3G)×100]. Results are mean±s.e.m. (n=4).

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hydrophobic (2L) or more hydrophobic (7L) constructs (data not shown). The range of hydrophobicity of the H3 segment that is sensitive to Sec62 mutations is similar to the H1 model protein, suggesting the membrane insertion of the H3 segment might be independent of the first two TM segments.

Taken together, results from all three sets of model proteins show that the C_{out} membrane orientation of marginally hydrophobic TM segments is reduced in 35DDD and 103DDD *sec62* mutant strains.

DISCUSSION

The function of Sec62 has long been regarded as being limited to post-translational translocation of secretory proteins in yeast, but there has been a lack of molecular and mechanistic details. In the present study, we systematically mutagenized Sec62 and assessed how these Sec62 mutants mediate translocation and membrane insertion of proteins with diverse sequence context (e.g. various degrees of hydrophobicity, number of TM segments, flanking charged residues and different membrane orientations). Our results show that mutations in the N-terminal cytosolic domain of Sec62 disrupt the interaction with Sec63 and lead to defects in translocation of moderately hydrophobic TM segments of single- as well as multi-spanning membrane proteins. Our earlier study has shown that the targeting of H1 model and Spc3 single-spanning membrane proteins is impaired in a yeast strain with a defective SRP, suggesting that these proteins are subject to co-translational translocation (Reithinger et al., 2013). Given that the first TM segment of H2 and H3 model proteins is more hydrophobic than TM segments of H1 and Spc3 single-spanning membrane proteins, it is likely that H2 and H3 model proteins are targeted by the SRP-dependent co-translational translocation, and indeed we observed that (data not shown). Hence, the defects observed in this study are due to the impaired function of the Sec62–Sec63 complex in translocation and membrane insertion of the test proteins in the ER membrane rather than targeting of those to the ER.

An earlier study by Plath et al. (Plath et al., 1998) revealed the mode of signal sequence binding using an elegant site-specific cross-linking approach. When a photoreactive lysine derivative was site-specifically incorporated into the residues of the signal sequence of prepro-α-factor (ppα), crosslinking between the ppα signal sequence, and Sec61 and Sec62–Sec71 occurred along the entire signal sequence, indicating that the signal-sequence-binding site is formed by Sec61 and Sec62–Sec71, and the signal sequence forms a helix perpendicular to the plane of the membrane (Plath et al., 1998; Plath et al., 2004). Interestingly, when the same experiment was performed with a photoreactive phenylalanine derivative that has a shorter linker than the lysine derivative, only the photo-probe located at the C-terminal end of the ppα signal sequence crosslinked to Sec62–Sec71, suggesting that the C-terminal end of the signal sequence interacts with Sec62–Sec71 (Plath et al., 1998). Given that hydrophobicity of signal peptides of post-translationally translocated proteins is moderate, and the sequence and structural context of moderately hydrophobic TM segments of multi-spanning membrane proteins are similar to those of signal peptide (Ng et al., 1996), it would not be surprising if Sec62 recognizes both. In light of our results, the previously observed translocation defect of post-translationally translocated proteins in Sec62 defective strains (Ng et al., 1996) could alternatively be explained by defective Sec62 failing to orientate the signal peptide correctly as N_{in}-C_{out} for cleavage in the ER membrane.

The Sec62–Sec63 translocon includes the non-essential Sec71 and Sec72 subunits. Sec71 and Sec72 were first discovered as genes that rescued defects in translocation of multi-spanning membrane proteins (Green et al., 1992). Although the function of Sec71 and Sec72 in membrane protein insertion remains elusive, the fact that they are in a complex with Sec62 and Sec63 further suggests a highly possible and general role of the Sec62–Sec63 translocon in membrane protein biogenesis.

In mammalian cells, a study by Lang et al. has shown that depletion of Sec62 or Sec63 leads to translocation defects for a subset of secretory proteins (Lang et al., 2012), suggesting that there is the functional similarity to the yeast Sec62–Sec63 complex. It remains to be seen whether the mammalian Sec62–Sec63 complex also handles insertion of multi-spanning membrane proteins. Further, the translocating-chain-associating membrane protein (TRAM) has been found in close proximity to Sec61 and functions in the early stage of translocation of some secretory and membrane proteins (Görlich et al., 1992; Görlich and Rapoport, 1993; High et al., 1993; Mothes et al., 1994; Do et al., 1996; Voigt et al., 1996; Heinrich et al., 2000; McCormick et al., 2003; Saurí et al., 2007). It has been shown that the features of the signal sequence dictate TRAM dependency (Voigt et al., 1996). TRAM-dependent secretory proteins appear to have signal sequences that have shorter N-terminal tails with charged residues and are less hydrophobic, thus it was proposed that TRAM might be involved in orienting the signal sequence correctly for cleavage by the signal peptidase (Voigt et al., 1996). Recently, Sommer et al. (Sommer et al., 2013) have reported that the translocon-associated protein (TRAP) complex is involved in membrane protein topogenesis in mammalian cells. Thus, it seems that the TRAM and TRAP complexes in mammalian cells have a functional resemblance to the Sec62–Sec63 translocon in yeast.

MATERIALS AND METHODS

Yeast strains

JRY4 (*MATα, sec62Δ::HIS3, ade2, can1, his3, leu2, trp1, ura3*, pRS416SEC62), a *SEC62* deletion strain, was generated by plasmid shuffling on a 5FOA agar plate and homologous recombination. First, the *SEC62* gene including the sequences 1.5 kb upstream was amplified using primers 5′-CGATAAGCTTGATATCGAATTCCTGCAGGGGTCTATC-TAGGATAC-3′ (RP25) and 5′-GGCGGCCGCTCTAGAACTAGTGG-ATCCGAGGTTACAATATAGAAGG-3′ (RP26) from the genomic DNA of W303-1α (*MATα, ade2, can1, his3, leu2, trp1, ura3*). The amplified gene was introduced into *Sma*I-cleaved pRS415 and pRS416 plasmids by homologous recombination (recombination sequences are underlined). The recombinant pRS416 plasmid was transformed into W303-1α. Then, genomic *SEC62* gene was replaced with the *HIS3* gene by homologous recombination. The *HIS3* gene was amplified using primers 5′-GGGAGAAGAGT-GGGCTTTTATAATTGCAGTTGAATGCAGTCACAGGAAACAGCTA-TGACC-3′ (RP27) and 5′-GAAGGTTTATACAGTAGAGCTATACAGG-ATAATGGAAGTGTTGTAAAACGACGGCCAGT-3′ (RP28) from the pCgH plasmid (recombination sequences are underlined) (Kitada et al., 1995). The resultant colonies were selected on agar plates without histidine (–His). JRY6 (*MATα, SEC63::HA-KanM, sec62Δ::HIS3, ade2, can1, his3, leu2, trp1, ura3*, pRS416SEC62) was generated by tagging the genomic *SEC63* with three copies of an *HA* epitope, and then following the same procedure used to make JRY4 to delete the *SEC62* gene. The cassette of the *HA* tag and selectable marker for kanamycine was amplified with primers 5′-CGATACGGATACAGAAGCTGAAGATGATGAATCACCAGAAC-GGATCCCCGGGTTAATTAA-3′ (RP70) and 5′-CTAAGAGCTAAAA-TGAAAAACTATACTAATCACTTATATCGAATTCGAGCTCGTTTA-AAC-3′ (RP71) from the pFA6a-3HA-kanMX6 plasmid (recombination sequences are underlined) (Longtine et al., 1998). The resulting PCR fragment was transformed into W303-1α, and cells were grown on YPD medium and replica-plated on a YPD-G418 plate.

Primer design

Forward and reverse primers contained 30 bases complementing the upstream and downstream sequences of the *Sma*I site in a p424GPDHA plasmid (Lundin et al., 2008) for homologous recombination and 17–20 bases complementing the start and end of the gene of interest. In the case of the Lep IV truncated model protein (Lep-H3), the reverse primer contained 17 bases complementing nucleotides 653–669 of Lep IV (Öjemalm et al., 2012).

Construction of plasmids

Using the oligonucleotide synthesized, genes of interest were amplified from the genomic DNA of W303-1α by colony PCR. All plasmids were constructed by homologous recombination as previously described (Lundin et al., 2008; Reithinger et al., 2013). Mutations and the FLAG epitope were introduced using a site-directed mutagenesis kit (Toyobo, Japan). For conjugation of the FLAG tag to SEC62 WT and *sec62* mutants in pRS415 plasmid, primer RP98 (5′-GTTTTGTTCGGCTTTTTCATTGATG-3′) and RP100 (5′-GACTACAAGGACGACGATGACAAGTGAACTTCCATTATCCTGTATAG-C-3′) were used. pHK131 DT-Spc3HA was constructed as described previously (Reithinger et al., 2013). The protein sequences are found in supplementary material Fig. S1.

Western blot analysis, pulse-labeling and immunoprecipitation

These experiments were carried out as described previously (Reithinger et al., 2013).

Co-immunoprecipitation

JRY6 cells expressing FLAG-tagged *SEC62* WT or mutant *sec62* were grown at 30℃ in medium without leucine and histidine (–Leu –His) until they reached an optical density at 600 nm (OD_{600}) of 1.0. A total of 10 OD_{600} unit cells were harvested at 3000 ***g*** and washed with distilled H_2O. The cell pellet was solubilized with 200 µl of lysis buffer (20 mM Tris-HCl pH 8, 10 mM EDTA, pH 8, 20 mM NaCl, 300 mM Sorbitol, 1 mM PMSF and protease inhibitor cocktail) and vortexed with prewashed glass beads for 10 min at 4℃. The lysate was centrifuged briefly to remove cell debris, and the supernatant was centrifuged at 28,000 ***g*** for 30 min at 4℃ to harvest the membrane fraction. The pellet was resuspended in 200 µl of lysis buffer (20 mM Tris-HCl, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 1 mM PMSF and protease inhibitor cocktail) and incubated on ice for 30 min. Of the resuspension, 20 µl of the reaction was taken out as an input sample. Another 150 µl of the resuspension was mixed with 500 µl of immunoprecipitation buffer A (15 mM Tris-HCl, 0.2% Trition X-100 and 100 mM NaCl), 3 µl anti-HA antibody, and 25 µl of protein-G–agarose beads, then the mixture was rotated overnight at 4℃. The agarose beads were washed once with immunoprecipitation buffer B (15 mM Tris-HCl, 0.4% Trition X-100 and 100 mM NaCl), twice with immunoprecipitation buffer A, and once with immunoprecipitation buffer C (15 mM Tris-HCl and 100 mM NaCl). Then, the washed beads were heated with 50 µl SDS-PAGE sample buffer at 65℃ for 15 min, and samples were then analyzed by SDS-PAGE and western blotting.

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Competing interests

The authors declare no competing interests.

Author contributions

S.J., J.K., J.H.R. and H.K. designed and analyzed the experiments, and wrote the manuscript. S.J. and J.K. performed the experiments.

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Supplementary material

Supplementary material available online at
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Supplementary Material

H1-4L

MANSTSQGSQPINAQTSGGPG**AAAALALAAAAALALAAAA**GPGGVPIPSGSMMPTLNSTDFILVEKFAYG
IKDPIYQKTLIENSTAKRGDIVVFKYPEDPKLDYIKRAVGLPGDKVTYDPVSKELTIQPGCSSGQACENA
LPVTYSNVEPSDFVQTFSRRNGGEATSGFFEVPKQETKENGIRLSERKETLGDVTHRILTVPIAQDQVGM
YYQQPGQQLATWIVPPGQYFMMGDNRDNSADSRYWGFVPEANLVGRATAIWMSFDKQEGEWPTGLRLSRI
GGIHLQPSYPYDVPDYAGYPYDVPDYAGSYPYDVPDYALLESCN*

H2-2L

MANSTSQGSQPINAQAAPVAQGGSQGE**FALILVIATLVTGILWCVD**KFFFAPKRRERQAAAQAAAGDSLD
KATLKKVAPKTSGGPG**AAAALAAAAAAAAALAAAA**GPGGVPIPSGSMMPTLNSTDFILVEKFAYGIKDPI
YQKTLIETGHPKRGDIVVFKYPEDPKLDYIKRAVGLPGDKVTYDPVSKELTIQPGCSSGQACENALPVTY
SNVEPSDFVQTFSRRNGGEATSGFFEVPKQETKENGIRLSERKETLGDVTHRILTVPIAQDQVGMYYQQP
GQQLATWIVPPGQYFMMGDNRDNSADSRYWGFVPEANLVGRATAIWMSFDKQEGEWPTGLRLSRIGGIHL
QPSYPYDVPDYAGYPYDVPDYAGSYPYDVPDYALLESCN*

H3-4L

MANSTSQGSQPINAQAAPVAQGGSQGE**FALILVIATLVTGILWCVD**KFFFAPKRRERQAAAQAAAGDSLD
KATLKKVAPKTSGGPG**ALAALALAALAALALAALA**GPGGVPIPSGSMMPTLNSTDFILVEAFAYGIADPI
YQATLIETGHPAPGELGGPG**AAAALALAAAAALALAAAA**GPGGLEYIAPAVGLPGDNVTYDPVSAELTIQ
PGCSSGQACENGPLQPSYPYDVPDYAGYPYDVPDYAGSYPYDVPDYALLESCN*

Fig. S1. Amino acid sequences of proteins used in this study. Predicted TM segments are shown in bold and the test TM segments are indicated in red. N-linked glycosylation sites are underlined.