Supplementary Information

Yeast strains. Mutations were introduced into the TIM15 gene by PCR-based mutagenesis to make genes for $Tim15^{5DE}$, $Tim15^{2RH}$, $Tim15^{D111A}$, and $Tim15^{\Delta 133\text{-}137}$. Yeast haploid strain (Δtim15/pRS316-Tim15) (Yamamoto et al, 2005), in which chromosomal disruption of the *TIM15* gene was complemented with a *URA3* plasmid harboring the *TIM15* gene, was transformed with a *TRP1* single-copy plasmid harboring the wild-type *TIM15* gene or the mutant *tim15* genes. Yeast strains dependent on mutated *tim15* was selected on 0.1% 5′-fluoroorotic acid (5′-FOA) plates.

Preparation of Tim15c. Tim15 (residues 43-174 lacking the presequence), Tim15 mutants, and Tim15c (residues 64-159) were overexpressed in *E. coli* strain BL21 (DE3) using the vector pET-15b(+) (Novagen). Briefly, cells were grown at 25°C to OD_{600} ~ 0.6, and induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside for 5 h at 25°C. Tim15 and Tim15c were purified on a Ni-NTA-agarose column (Qiagen) in the absence of Zn^{2+} according to the manufacturer's instruction. The His-tag of Tim15 and Tim15c was cleaved with thrombin, and Tim15c was further purified on a Mono-S column in the presence of 30 μM $ZnSO_4$ or $Zn(OAC)_2$. For preparation of uniformly ^{15}N-labeled or $^{15}N/^{13}C$-labeled Tim15 and Tim15c, cells were grown in M9 minimal medium containing 30 μM $Zn(OAC)_2$, supplemented with $^{15}NH_4Cl$ (1 g/l) and/or [U-^{13}C]-glucose (2 g/l), respectively.

Preparation of Yge1p(Mge1p). Yge1p without presequence was overexpressed in *E. coli* strain BL21 (DE3) using the vector pET-21a(+) (Novagen). Briefly, cells were grown at 37°C to OD_{600} ≈ 0.6, and induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C. Yge1p was purified on a Ni-NTA-agarose column (Qiagen).

NMR structural determination. Distance constraints for the structure determination were obtained from 3D ^{15}N-edited [^1H, ^1H]-NOESY (H_2O), 3D ^{13}C-edited [^1H, ^1H]-NOESY (D_2O) and 2D [^1H, ^1H]-NOESY (D_2O) spectra of Tim15c with a mixing time of 150 ms. Slowly exchanging amide protons were identified by acquiring a series of [^1H, ^{15}N]-HSQC spectra after dissolving the lyophilized protein in 99.9% D_2O. Structure calculations were performed using the program CYANA (Güntert *et al*, 1997). A final set of NOE restraints was supplemented with 100 torsion angle restraints for ϕ and ψ angles, which were obtained from TALOS (Cornilescu *et al*, 1999), 36 hydrogen-bond restraints derived from HD exchange experiments and 12 Zn^{2+}-binding restraints for residues involved in direct Zn^{2+} binding as S_γ-S_γ distances (3.4-4 Å). The final ensemble of 20 lowest-energy refined structures was used to calculate the mean coordinate positions. The statistics are summarized in Table 1. Data analyses, structure display, and figure drawing were carried out using the program PyMOL.

Miscellaneous. Co-expression of mtHsp70 and Tim15 derivatives or Yge1p in *E. coli* was carried out by using the pETDuet-1 system (Novagen) (Sichting et al, 2005). Circular dichroism (CD) spectra of Tim15 and its mutants were recorded in CD buffer

(20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM DTT, 30 μM $ZnSO_4$) on a JASCO J-720 spectropolarimeter, at 25°C, using a 0.2 cm path-length cell.

For the pull-down assay with GST-fusion proteins, GST and GST-Tim15 were expressed from a pGEX4T2 plasmid in the *E. coli* strain BL21 (DE3). Ssc1p (mtHsp70, without a presequence) was co-expressed with trigger factor in the *E. coli* strain BL21 (DE3) using the vector pCold2 (Takara Bio.). Briefly, cells were grown at 37°C to OD_{600} ~ 0.6, shifted to16°C for 30 min, and induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside for 24 h at 16°C. Ssc1p was purified on a Ni-NTA agarose column (Qiagen) and subsequently on a mono-Q column in the presence of EDTA. After purifications, Ssc1p was dialyzed twice against 20 mM Tris-HCl, pH 7.4, 0.1 M KCl at 4°C to remove EDTA. 1 μM GST and GST-Tim15 were incubated with mtHsp70 (1μM), which had been extensively dialyzed against nucleotide-free buffer, in binding buffer (20 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM DTT, 30 μM $ZnSO_4$) containing 100 μl of glutathione-Sepharose (Amersham) in the presence or absence of 2 mM $MgCl_2$ and 1 mM ADP, ATP-γS, or AMP-PNP for 30 min at 25°C. The resin was then washed with binding buffer with or without 1 mM ADP, ATP-γS, or AMP-PNP and the bound proteins were eluted with 20 mM glutathione.

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Yamamoto H, Momose T, Yatsukawa Y, Ohshima C, Ishikawa D, Sato T, Tamura Y, Ohwa Y, Endo T (2005) Identification of a novel member of yeast mitochondrial Hsp70-associated motor and chaperone proteins that facilitates protein translocation across the inner membrane. *FEBS Lett* **579:** 507-511

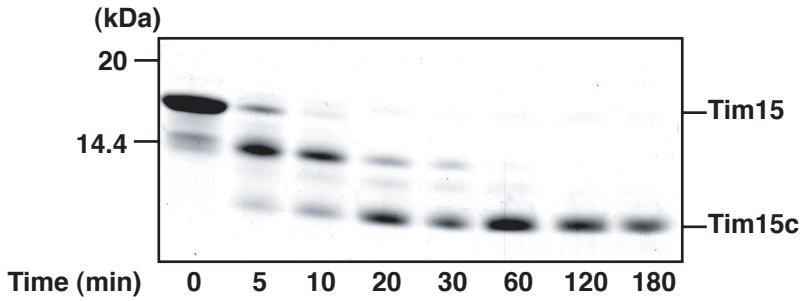

Fig 1 Limited digestion of Tim15 with trypsin. Tim15 (residues 43-174, 0.5 mg/ml) was digested with 25 mg/ml trypsin for indicated time on ice (in 20 mM Hepes-KOH, pH 7.4, 50 mM KCl, 1 mM DTT, 30 μM $Zn(OAc)_2$). With increasing time of trypsin digestion, Tim15 with apparent MW of 16.7 kDa was converted to a smaller fragment of 14.0 kDa, and subsequently to a protease-resistant fragment of 10.3 kDa, which was named Tim15c. Precise determination of the molecular mass of Tim15c as 10,894 by mass-spectrometry analyses revealed that it corresponds to a tryptic fragment of residues 64-159 of Tim15.

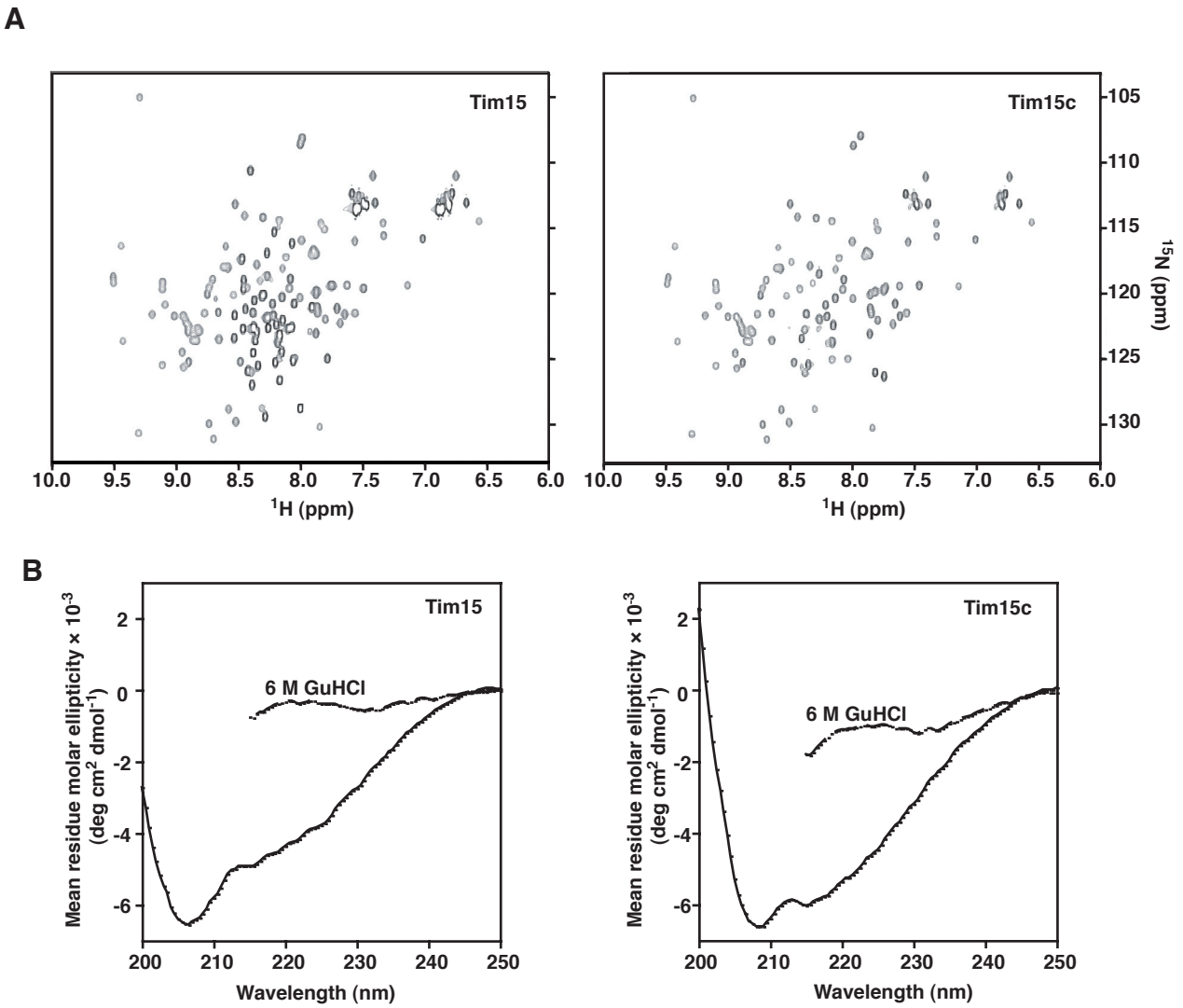

Fig 2 Comparison of ^{1}H, ^{15}N-HSQC spectra and CD spectra of Tim15 and Tim15c. (A) ^{1}H, ^{15}N-HSQC spectra of 0.2 mM Tim15 (left) and Tim15c (right) in 20 mM sodium acetate, pH 4.5, 50 mM NaCl, 1 mM DTT, 30 µM $Zn(OAc)_2$, 95% H_2O/5%D_2O at 299K. (B) Far-UV CD spectra of 5 µM Tim15 (left) and Tim15c (right) in CD buffer at 25°C.

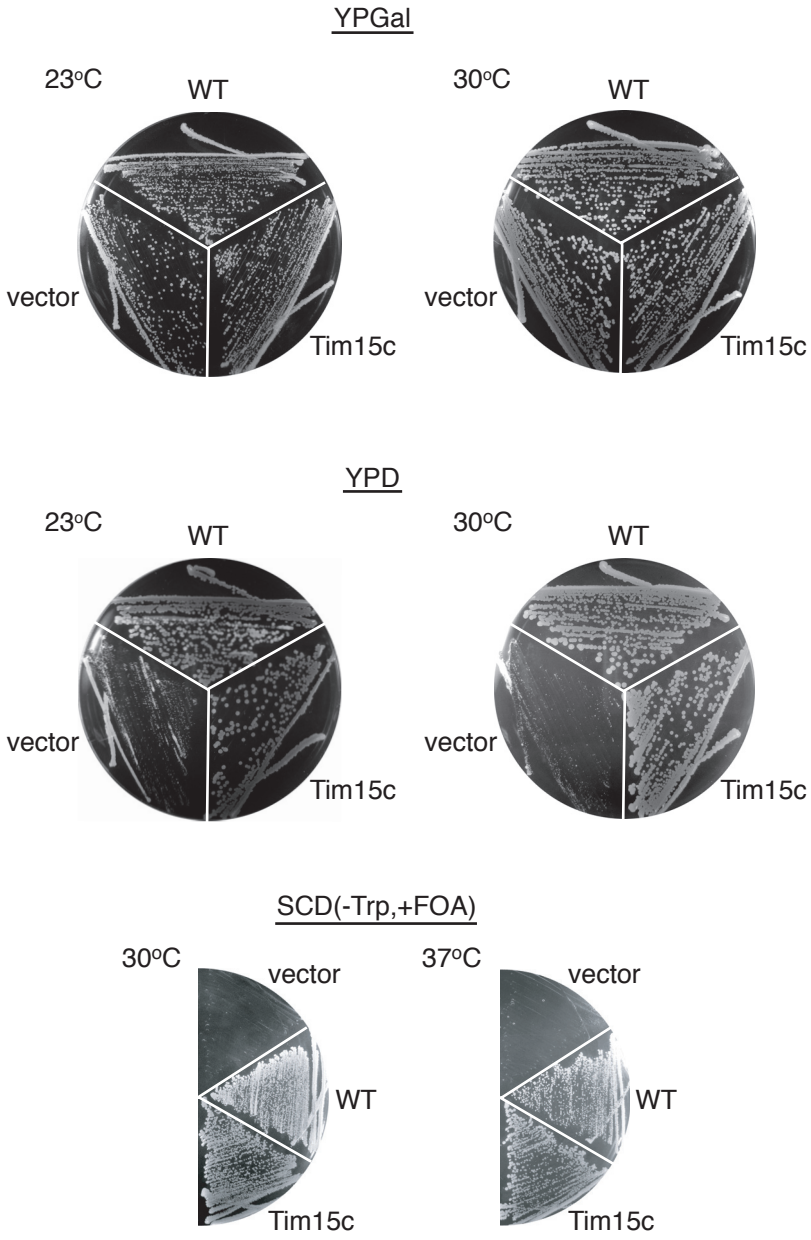

Fig 3 Tim15c can replace Tim15 in yeast cells. Plasmids (pRS316) carrying the genes for wild-type Tim15 (WT) or Tim15c, or without an inserted gene (vector) were introduced into the GAL-TIM15 strain. The resulting transformants were grown on YPGal plates (upper panel) and YPD plates (center panel) at 23°C or 30°C for 2 days (30°C) or 3 days (23°C). Plasmids (pRS314) carrying the genes for wild-type Tim15 (WT) or Tim15c, or without an inserted gene (vector) were introduced into the tim15Δ strain supplied with the TIM15 gene from a single-copy plasmid of the URA3 selection marker (Δtim15/pRS316-Tim15). The resulting transformants were grown on SCD(-Trp) plates containing 0.1% 5-FOA at 30°C for 3 days or 37°C for 2 days (lower panel).

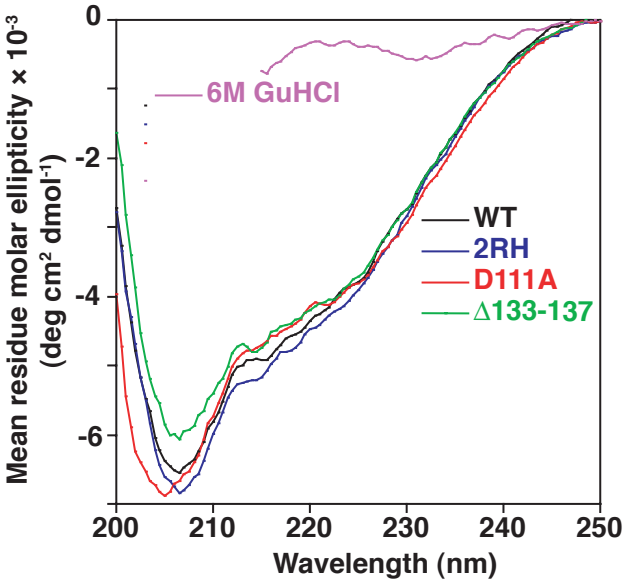

Fig 4 Mutations of 2RH, D111A or Δ133-137 do not affect the overall folding of Tim15. Far-UV CD spectra of wild-type 5 mM Tim15 (WT, black), $Tim15^{2RH}$ (2RH, blue), $Tim15^{D111A}$ (D111A, red), and $Tim15^{\Delta 133\text{-}137}$ (Δ133-137, green) in CD buffer at 25°C. A CD spectrum of Tim15 denatured with 6 M GuHCl (magenta) is also shown. Recombinant $Tim15^{2RH}$, $Tim15^{D111A}$, and $Tim15^{\Delta 133\text{-}137}$ were purified from *E. coli* cells.

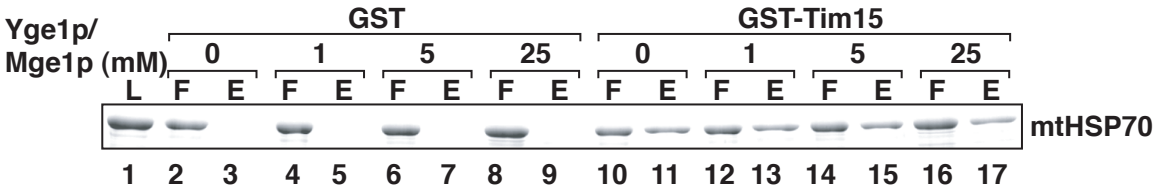

Fig 5 GST-Tim15 does not compete with Yge1p/Mge1p in binding to mtHsp70. Co-purification of nucleotide-free mtHsp70 with GST-Tim15 by glutathione-Sepharose was performed as in Fig. 4A in the presence of increasing concentrations (0, 1, 5, 25 μM) of Yge1p/Mge1p.