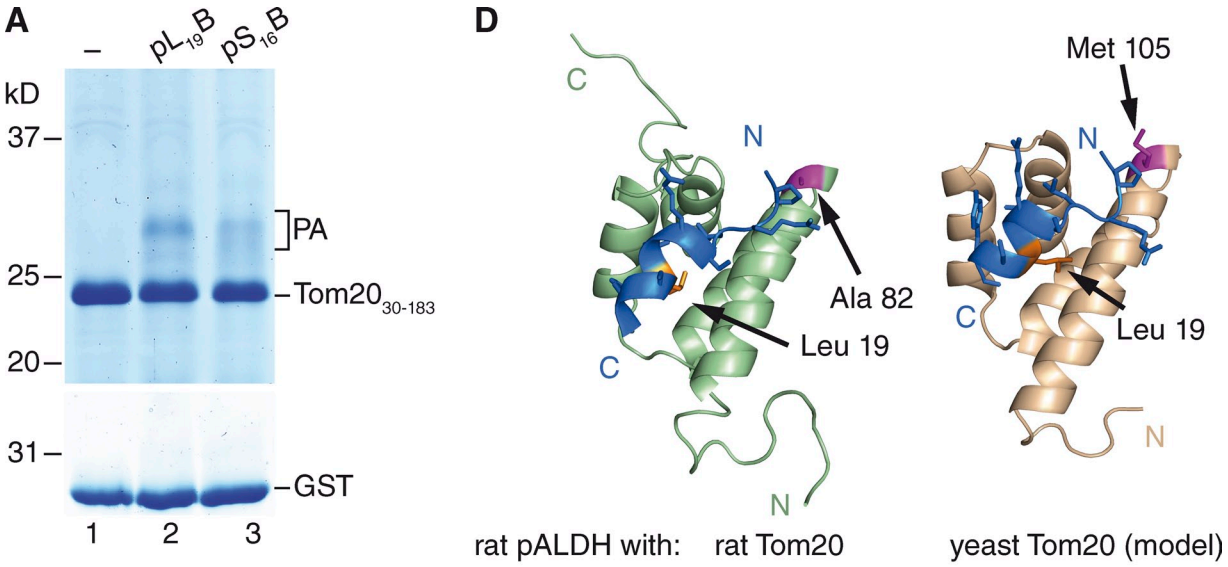
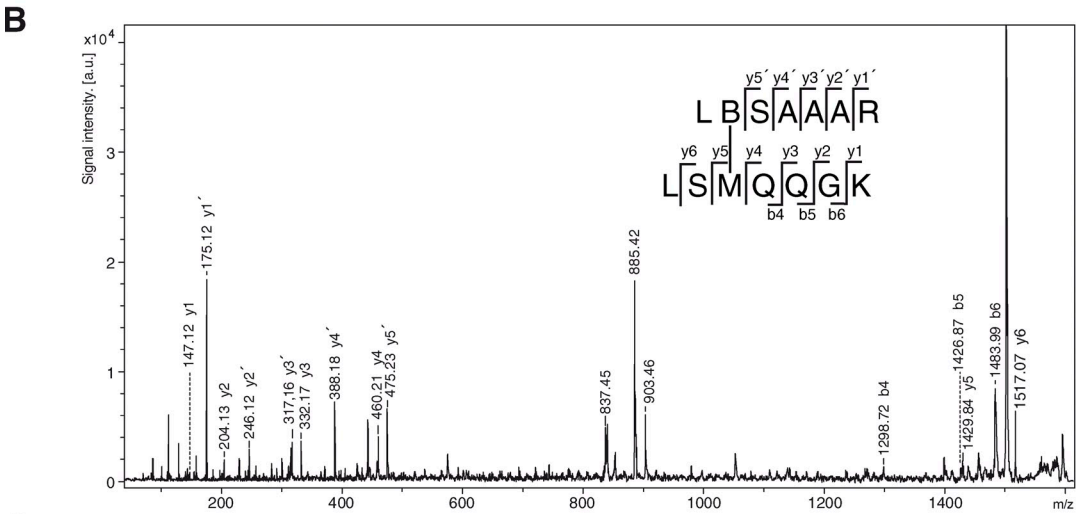
Schulz et al., http://www.jcb.org/cgi/content/full/jcb.201105098/DC1





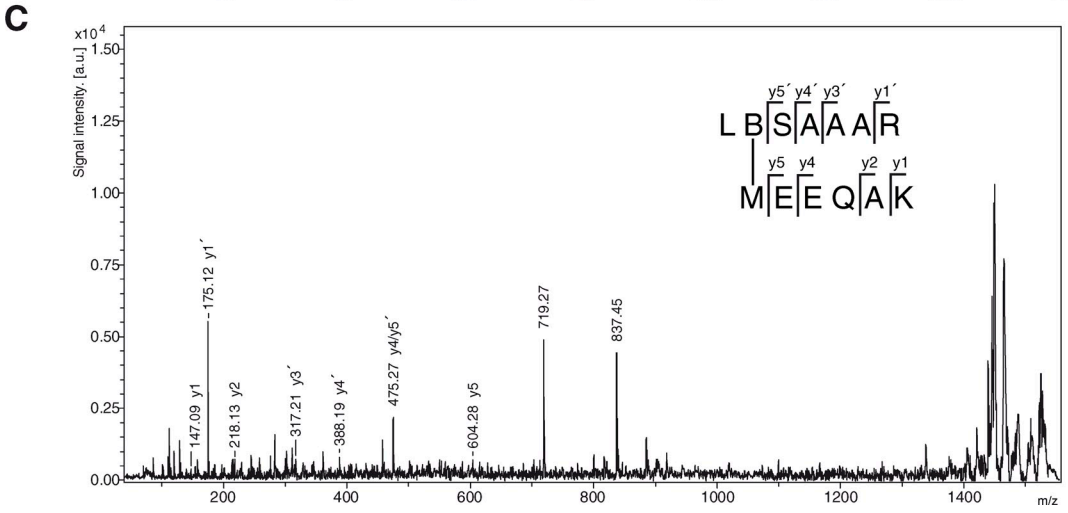


Figure S1. **Validation of the approach using Tom20^{CD}.** (A) Isolated Tom20^{CD} and GST were incubated with presequence probes under UV irradiation, samples analyzed by SDS-PAGE, and proteins subjected to in-gel digest before LC MALDI MS/MS analysis. PA, photo-adduct. (B) Fragment ion mass spectrum of peptide Tom20$^{53\text{-}58}$ cross-linked to pL$_{19}$B$^{18\text{-}24}$ (precursor mass 1573.75). Photo-adduct of pL$_{19}$B with Tom20^{CD} (A) was digested with trypsin and subjected to LC MALDI MS/MS analysis. (C) Fragment ion mass spectrum of peptide Tom20$^{103\text{-}109}$ cross-linked to pL$_{19}$B$^{18\text{-}24}$ (precursor mass 1629.84). Sample was analyzed as in B. (D) Representative model of the NMR structure of rat Tom20 in complex with pALDH presequence peptide (Protein Data Base accession no. 1OM2; Abe et al., 2000), left, and modeled yeast Tom20 in complex with pALDH (blue), right. Leu^{19} of pALDH and Ala^{82} or Met^{105} are colored in orange or magenta, respectively. N and C denote the N and C termini.

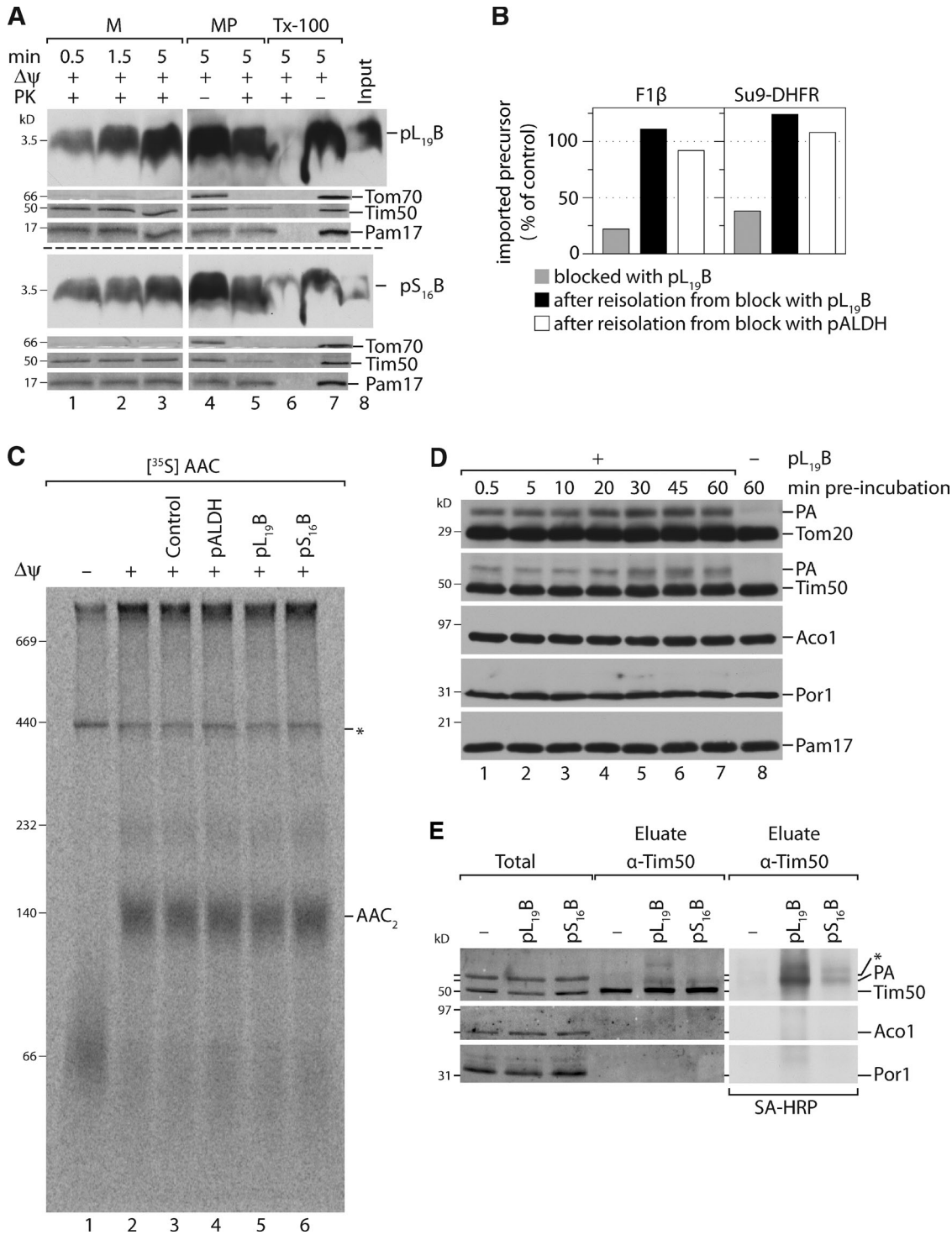


Figure S2. **Characterization of the photo-peptides.** (A) Presequence-probes $pL_{19}B$ (top panels) or $pS_{16}B$ (bottom panels) were imported into isolated mitochondria in the presence of $\Delta\psi$ for indicated times at 25°C. After import, mitochondria were converted into mitoplasts or resuspended in Triton X-100 containing buffer and subjected to proteinase K digestion. Samples were analyzed by Western blotting using streptavidin-HRP (horseradish peroxidase) or indicated antibodies. Input, 25% of peptide used for each time point. M, mitochondria. MP, mitoplasts. Tx-100, Triton X-100. (B) Mitochondria were incubated for 10 min with 2 μM presequence peptide in the presence or absence of $\Delta\psi$. After re-isolation of mitochondria, protein import of a radiolabeled precursor protein (F1β and Su9-DHFR) was assessed. Therefore, the radiolabeled precursor was added to the treated or untreated mitochondria and import performed for 15 min. Subsequent to proteinase K treatment, samples were analyzed by SDS-PAGE and digital autoradiography. Imported precursor was quantified relative to untreated mitochondria (100%). (C) Radiolabeled AAC was imported into isolated mitochondria in the presence of 2 μM of the indicated peptides for 45 min. After proteinase K treatment, mitochondria were solubilized and samples analyzed by BN-PAGE and digital autoradiography. Asterisk denotes unspecific band. (D) Isolated mitochondria were preincubated with 2 μM $pL_{19}B$ for the indicated time on ice before subjecting the samples to UV irradiation for 10 min. Samples were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. PA, photo-adduct. (E) Tim50 immunoprecipitation from mitochondria after photo cross-linking with 2 μM photo-peptides for 30 min. Samples were analyzed by Western blotting with indicated antibodies or streptavidin-HRP, SA-HRP. Asterisk denotes cross-reactive protein. Total, 5%; Eluate, 100%. PA, photo-adduct.

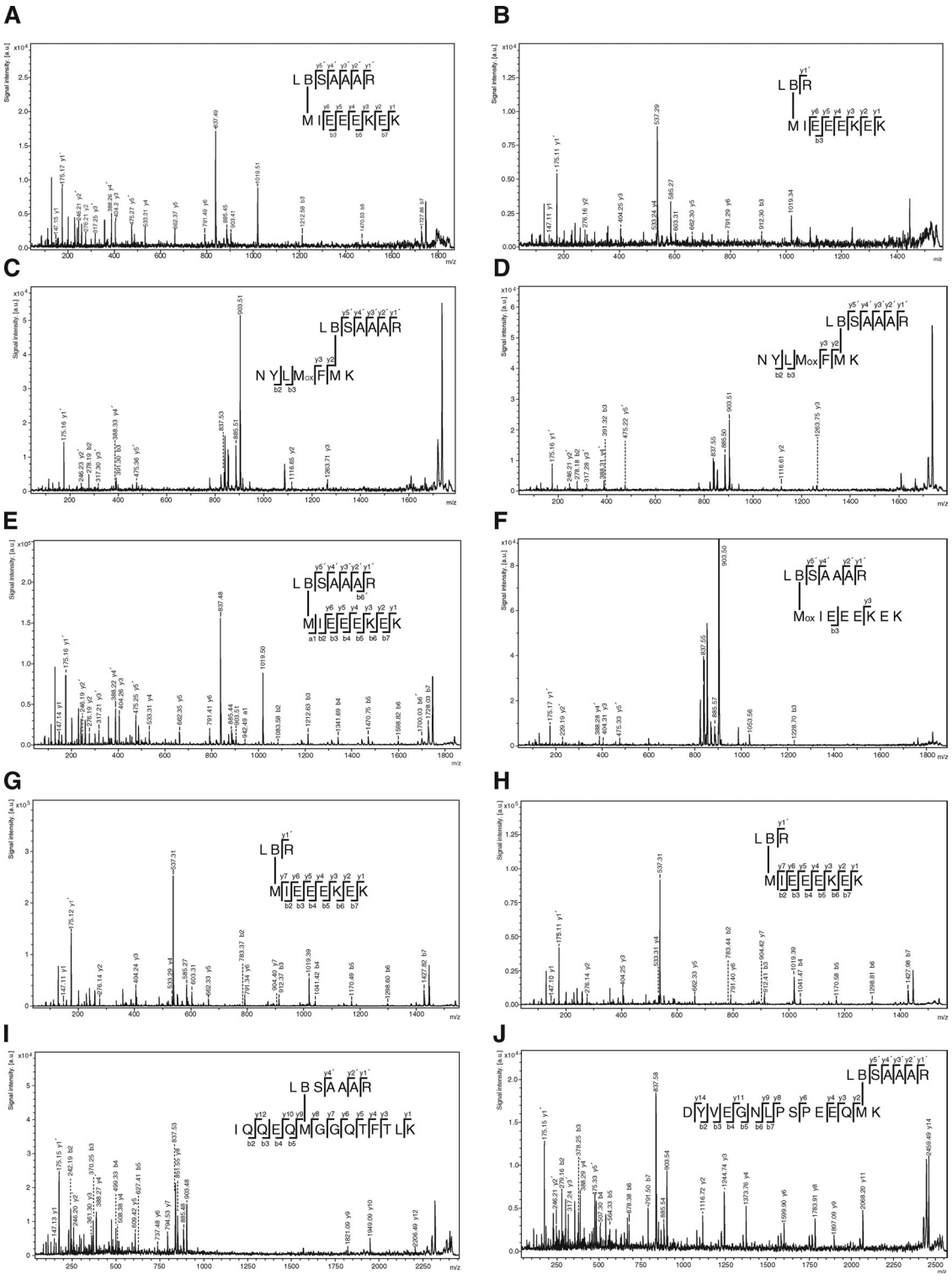


Figure S3. **Identification of the presequence-binding site in Tim50.** (A) After UV-induced cross-linking of $Tim50^{IMS}$ with peptide $pL_{19}B$ or $pS_{16}B$ and purification by SDS-PAGE, the lower bands containing photo-adducts (Fig. 2 B) were digested with trypsin and the peptides subjected to LC MALDI MS/MS analysis. The a-, b-, and y-ion series are indicated. Peptide $Tim50^{412\text{-}419}$ cross-linked to peptide $pL_{19}B^{18\text{-}24}$ (precursor mass 1873.90). (B) Peptide $Tim50^{412\text{-}419}$ cross-linked to peptide $pL_{19}B1^{8\text{-}24}$ (precursor mass 1573.78). Sample was analyzed as in A. (C–J) After UV-induced cross-linking of $Tim50^{PBD}$ with peptide $pL_{19}B$ or $pS_{16}B$ and purification by SDS-PAGE, bands corresponding to photo-adducts (Fig. 2 F) were digested with trypsin and the peptides subjected to LC MALDI MS/MS analysis. Where needed, lower and upper indicate the band excised from the gel and analyzed. The a-, b-, and y-ion series are indicated. (C) Peptide $Tim50^{405\text{-}411}$ cross-linked to peptide $pL_{19}B^{18\text{-}24}$ (precursor mass 1800.86); lower. (D) Peptide $Tim50^{405\text{-}411}$ cross-linked to peptide $pL_{19}B^{18\text{-}24}$ (precursor mass 1800.89); upper. (E) Peptide $Tim50^{412\text{-}419}$ cross-linked to peptide $pL_{19}B^{18\text{-}24}$ (precursor mass 1873.91); lower. (F) Peptide $Tim50^{412\text{-}419}$ cross-linked to peptide $pL_{19}B^{18\text{-}24}$ (precursor mass 1889.92); upper. (G) Peptide $Tim50^{412\text{-}419}$ cross-linked to peptide $pS_{16}B^{15\text{-}17}$ (precursor mass 1573.86); lower. (H) Peptide $Tim50^{412\text{-}419}$ cross-linked to peptide $pS_{16}B^{15\text{-}17}$ (precursor mass 1573.81); lower. (I) Peptide $Tim50^{422\text{-}435}$ cross-linked to peptide $pL_{19}B^{18\text{-}24}$ (precursor mass 2447.25). (J) Peptide Tim50436-450 cross-linked to peptide pL19B18-24 (precursor mass 2574.23); lower.

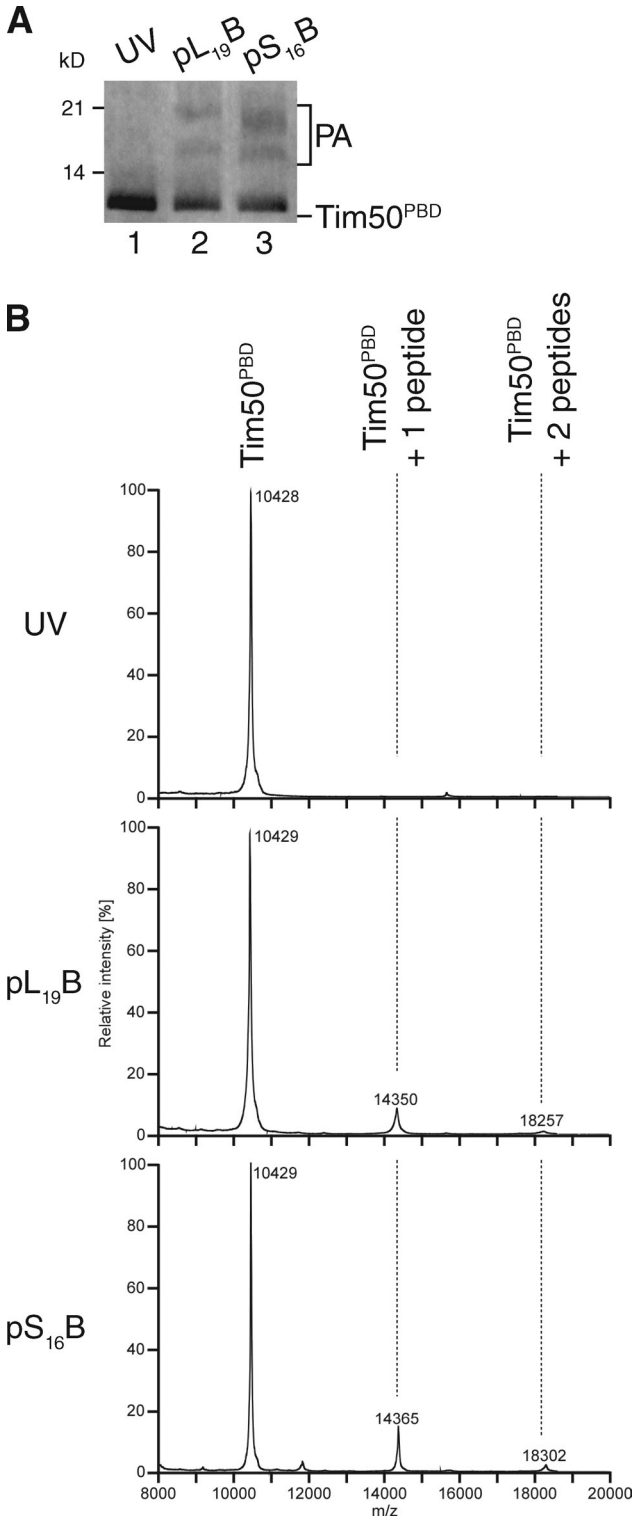


Figure S4. **Photo-adducts to Tim50^{PBD} contain mainly one presequence peptide.** (A) After UV-induced cross-linking of purified Tim50^{PBD} with $pL_{19}B$ or $pS_{16}B$, for 30 min as in Fig. 2 F, the sample was analyzed by Western blotting using anti-Tim50 antibodies. PA, photo-adduct. (B) Samples from A were analyzed by linear MALDI-TOF-MS. The calculated molecular masses are as follows: M_{calc}(Tim50^{PBD}) = 10428 Da; M_{calc}(Tim50^{PBD}+1$pL_{19}B$) = 14338 Da; M_{calc}(Tim50^{PBD}+1$pS_{16}B$) = 14364 Da; M_{calc}(Tim50^{PBD}+2$pL_{19}B$) = 18247 Da; M_{calc}(Tim50^{PBD}+2$pS_{16}B$) = 18299 Da. Observed shifts toward higher masses are most likely due to methionine oxidation. The relative areas of the relevant signals are 80.8, 17.8, and 1.4% for $pL_{19}B$; and 83.4, 14.6, and 2.0% for $pS_{16}B$.

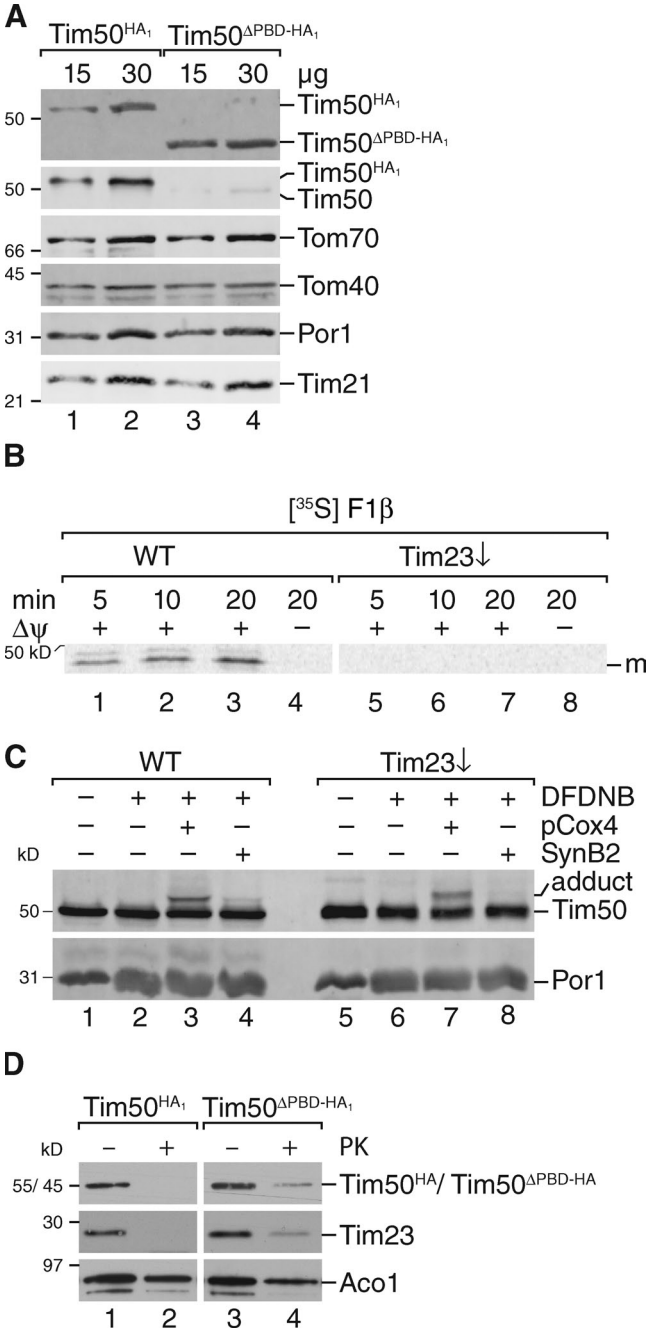


Figure S5. **Properties of different mutant mitochondria.** (A) Steady-state protein analysis of isolated Tim50↓ mitochondria expressing either Tim50^{HA1} or Tim50$^{\Delta PBD\ HA1}$ analyzed by Western blotting with the indicated antibodies. (B) Radiolabeled F1β was imported into wild-type and Tim23↓ mitochondria for the indicated times at 25°C. Subsequent to proteinase K digestion samples were analyzed by SDS-PAGE and digital autoradiography. m, mature protein. (C) Isolated mitochondria were incubated with 20 µM of the indicated peptides and subjected to chemical cross-linking using 1 mM DFDNB for 30 min. Samples were analyzed as in A. (D) Isolated Tim50↓ mitochondria expressing either Tim50^{HA1} or Tim50$^{\Delta PBD\ HA1}$ were converted into mitoplasts and subjected to proteinase K treatment. Samples were analyzed as in A.

Reference

Abe, Y., T. Shodai, T. Muto, K. Mihara, H. Torii, S. Nishikawa, T. Endo, and D. Kohda. 2000. Structural basis of presequence recognition by the mitochondrial protein import receptor Tom20. *Cell*. 100:551–560. http://dx.doi.org/10.1016/S0092-8674(00)80691-1