

Current Biology, Volume 30

Supplemental Information

**Defining the Substrate Spectrum
of the TIM22 Complex Identifies Pyruvate
Carrier Subunits as Unconventional Cargos**

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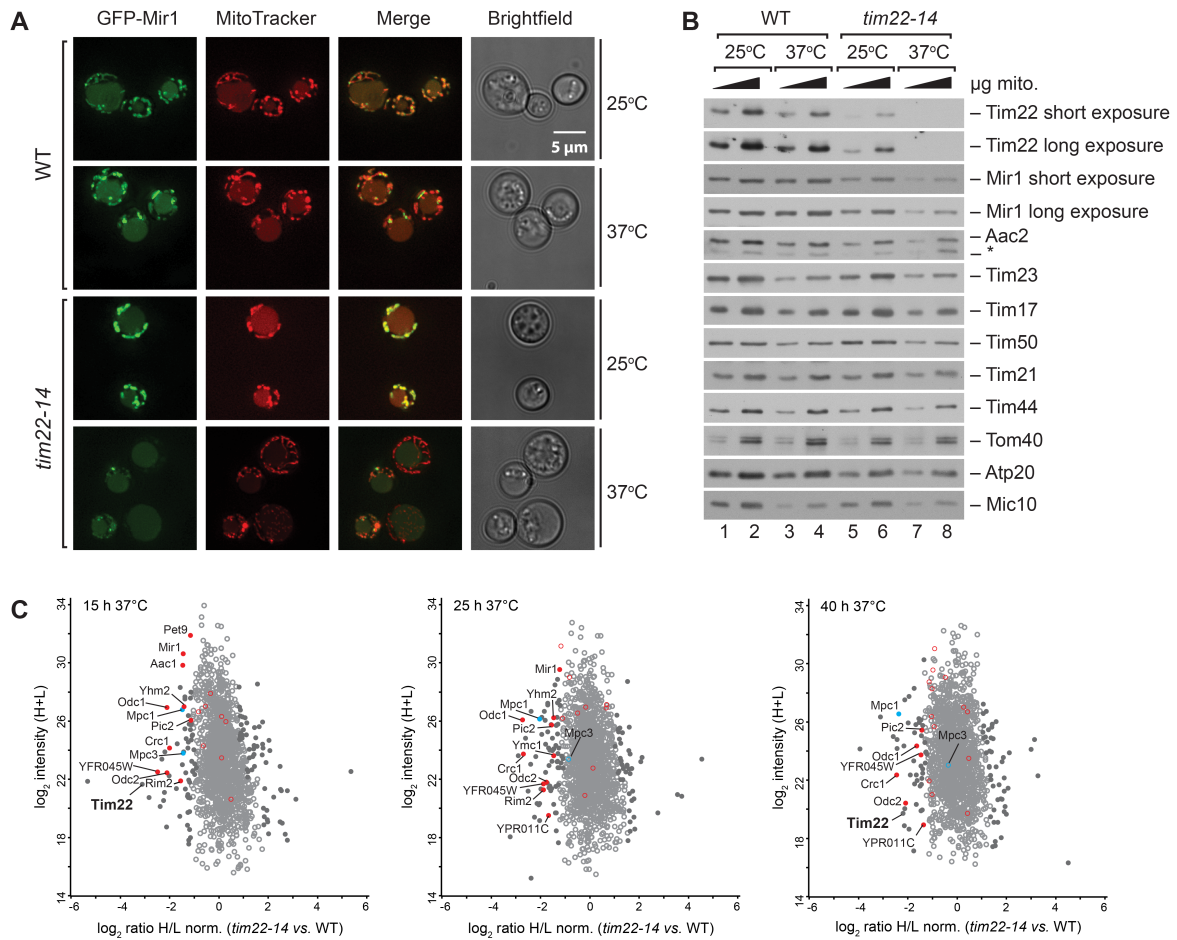


Figure S1. Carrier protein import is reduced in temperature sensitive Tim22 mutant, Related to Figure 1. (A) Wild type (WT) and *tim22-14* yeast cells expressing GFP-Mir1 were grown at 25°C or shifted to 37°C for 25 h. Cells were co-stained with MitoTracker Orange and analyzed by fluorescence microscopy. Merged green and red fluorescence images are shown (yellow/orange). (Scale bar: 5 µm). (B) Purified mitochondria from wild type (WT) and *tim22-14* cells grown at the permissive temperature (25°C) or shifted to the non-permissive temperature (37°C) for 14 h were analyzed by SDS-PAGE and western blotting. *: non-specific band. (C) Proteomic analyses of *tim22-14* versus WT mitochondria. log₂ ratio-intensity plots showing the effect of loss of Tim22 function on the abundance of mitochondrial carrier proteins (red) and MPC subunits (blue) after 15 h (i), 25 h (ii) and 40 h (iii) at 37°C. Filled circles indicate proteins significantly altered in abundance in each dataset.

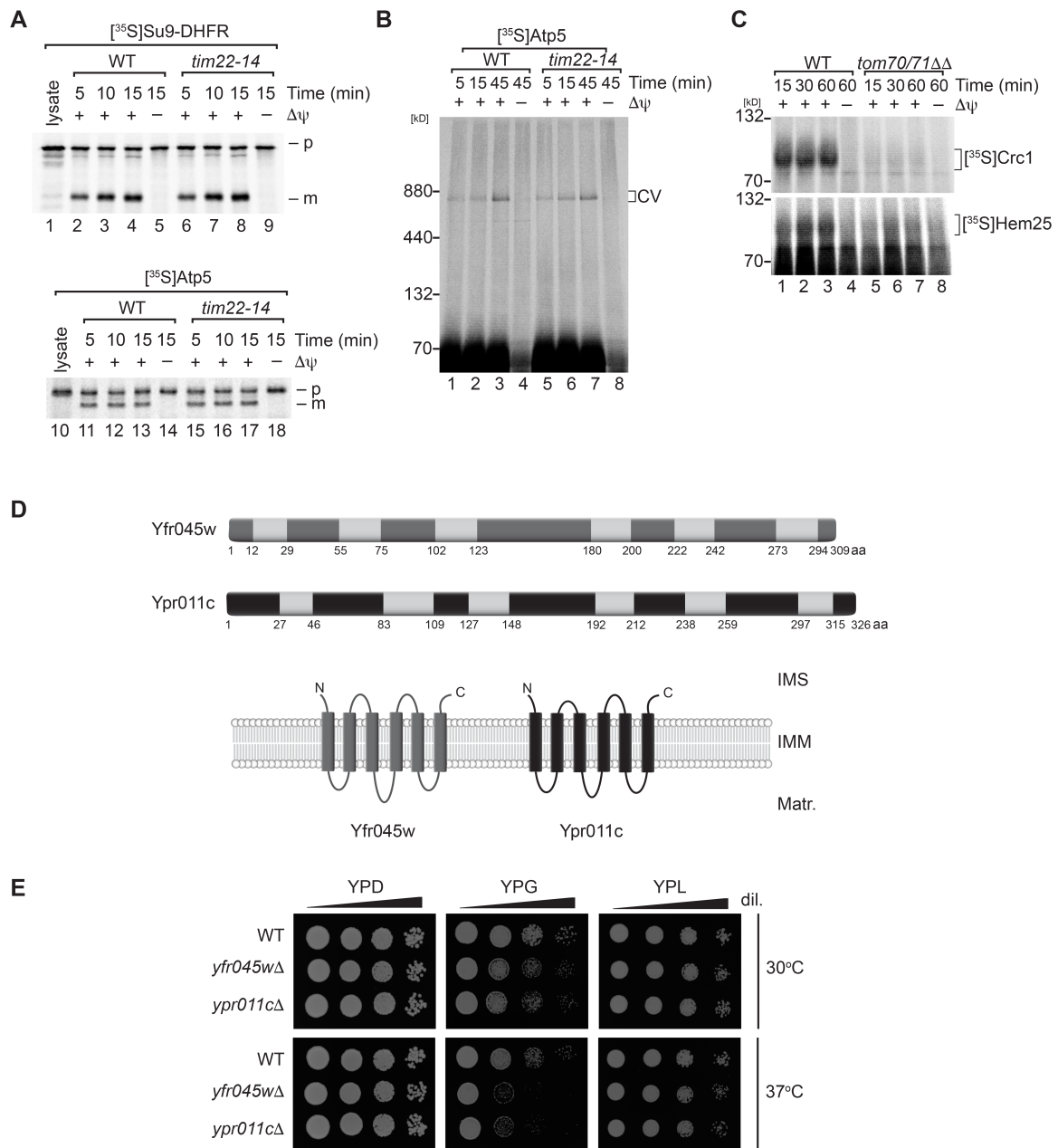


Figure S2. Presequence-containing precursor import is normal in *tim22-14* mitochondria, Tom70/71-dependent import of carrier proteins Crc1 and Hem25, and uncharacterized proteins Yfr045w and Ypr011c are required for mitochondrial activity, Related to Figure 2. (A) $[^{35}\text{S}]$ -labelled Su9-DHFR and Atp5 were imported into purified wild type (WT) and *tim22-14* mitochondria in the presence or absence of a membrane potential. Samples were analyzed by SDS-PAGE and digital autoradiography. p: precursor; m: mature. **(B)** $[^{35}\text{S}]$ -labelled Atp5 was imported in the presence or absence of a membrane potential into wild type (WT) and *tim22-14* mitochondria. After Proteinase K treatment, assembly of Atp5 into complex V was analyzed by BN-PAGE followed by digital autoradiography. **(C)** $[^{35}\text{S}]$ -labelled Crc1 and Hem25 were imported into purified wild type (WT) and *tom70/71ΔΔ* mitochondria in the presence or absence of a membrane potential. After Proteinase K treatment and solubilization of the samples, proteins were separated by BN-PAGE followed by digital autoradiography. **(D)** (*top*) Representation of the predicted transmembrane organization of Yfr045w and Ypr011c. Membrane topology prediction (*bottom*) based on the typical six transmembrane span arrangement of the carrier family. **(E)** Growth test of wild type (WT), *yfr045wΔ*, and *ypr011cΔ* cells on glucose (YPD), glycerol (YPG), or lactate (YPL) medium. N: N-terminus; C: C-terminus; aa: amino acid; IMM: inner mitochondrial membrane; IMS: intermembrane space; Matr.: matrix; dil.: dilution; $\Delta\psi$: membrane potential.

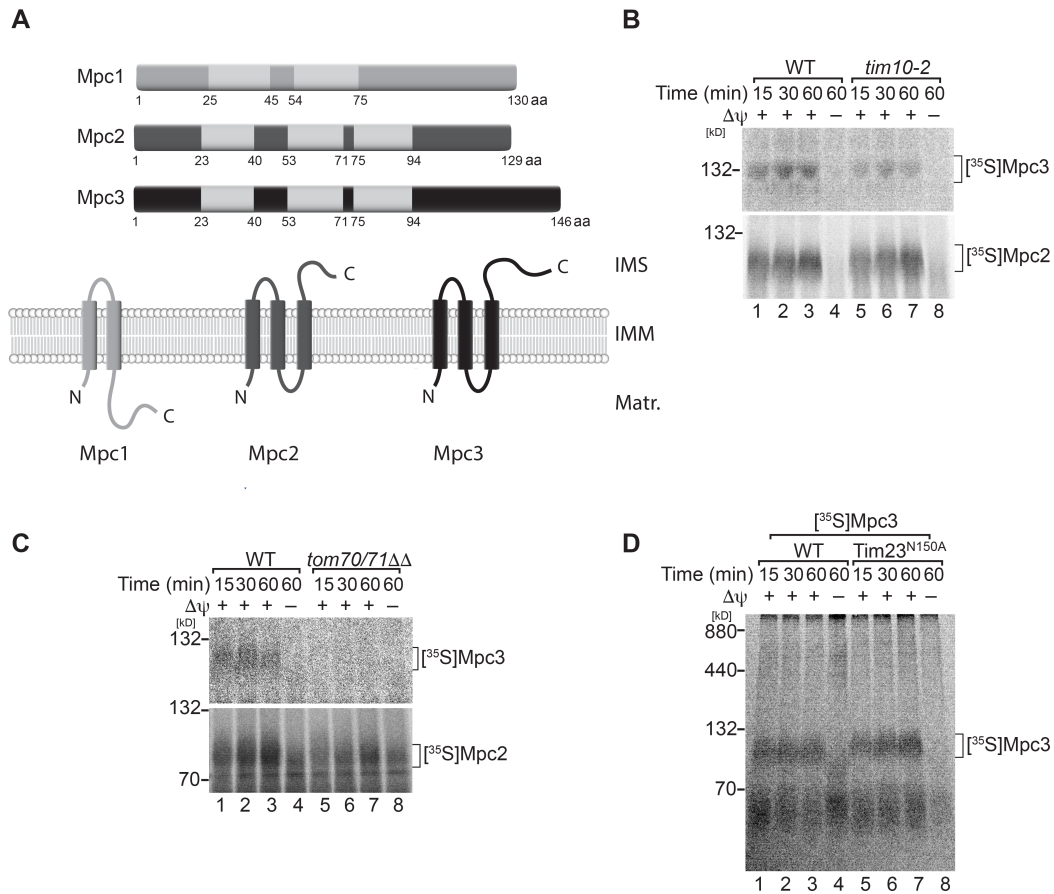


Figure S3. Mpc2 and Mpc3 import is dependent on Tom70/71 and Tim10, Related to Figure 3. (A) Representation of predicted transmembrane segments and topology of mitochondrial pyruvate carrier subunits Mpc1, Mpc2 and Mpc3. **(B)** [³⁵S]-labelled Mpc3 and Mpc2 were imported into wild type (WT) and *tim10-2* mitochondria. **(C)** [³⁵S]-labelled Mpc2 and Mpc3 were imported into purified wild type (WT) and *tom70/71* $\Delta\Delta$ mitochondria in the presence or absence of a membrane potential. **(D)** [³⁵S]-labelled Mpc3 was imported into wild type (WT) and *Tim23*^{N150A} mutant mitochondria in the presence or absence of a membrane potential. After Proteinase K treatment and solubilization of the samples, proteins were separated by BN-PAGE followed by digital autoradiography. N: N-terminus; C: C-terminus; aa: amino acid; IMM: inner mitochondrial membrane; IMS: intermembrane space; Matr.: matrix; $\Delta\psi$: membrane potential.