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RESEARCH ARTICLE

Figures and figure supplements

Protein translocation channel of mitochondrial inner membrane and matrix-exposed import motor communicate via two-domain coupling protein

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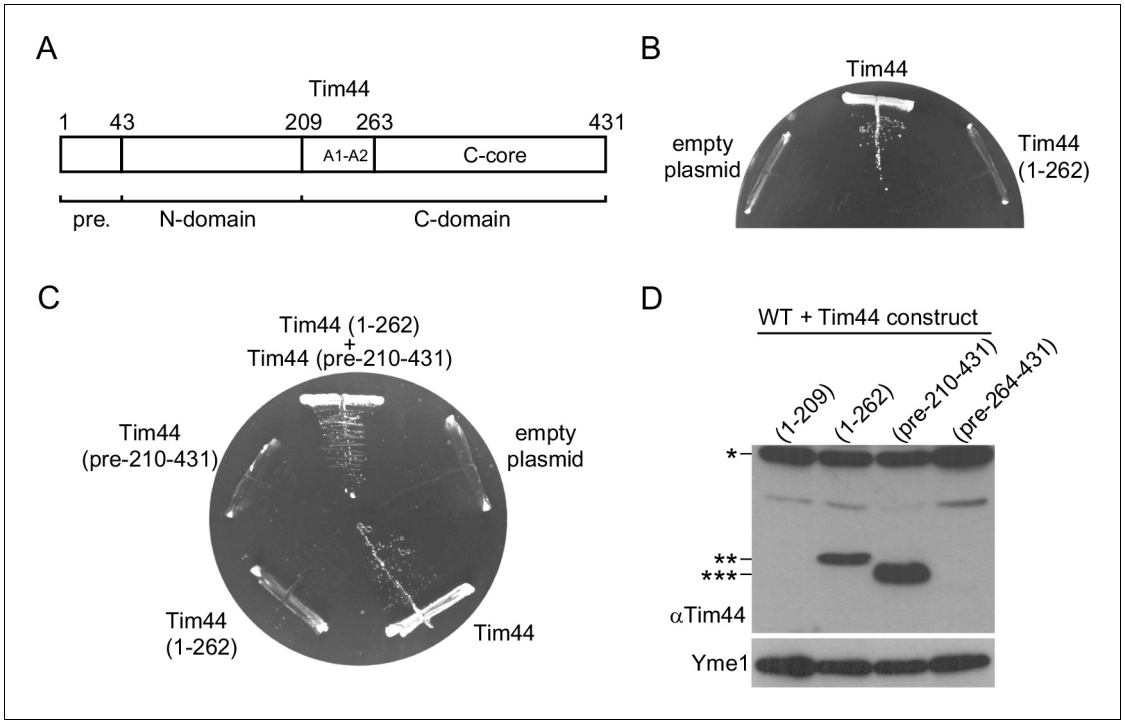

Figure 1. The function of Tim44 can be rescued by its two domains expressed *in trans* but not by either of the domains alone. (**A**) Schematic representation of Tim44 domain structure (numbering according to yeast Tim44 sequence). pre. - presequence (**B** and **C**) A haploid yeast deletion strain of *TIM44* carrying the wild-type copy of *TIM44* on a *URA* plasmid was transformed with centromeric plasmids carrying indicated constructs of Tim44 under control of endogenous promoter and 3'UTR. Cells were plated on medium containing 5-fluoroorotic acid and incubated at 30˚C. The plasmid carrying wild-type Tim44 and an empty plasmid were used as positive and negative controls, respectively. (**D**) Total cell extracts of wild-type yeast cells transformed with plasmids coding for indicated Tim44 constructs under *GPD* promoter were analysed by SDS–PAGE and immunoblotting against depicted antibodies. *, ** and *** - protein bands detected with antibodies raised against full-length Tim44.
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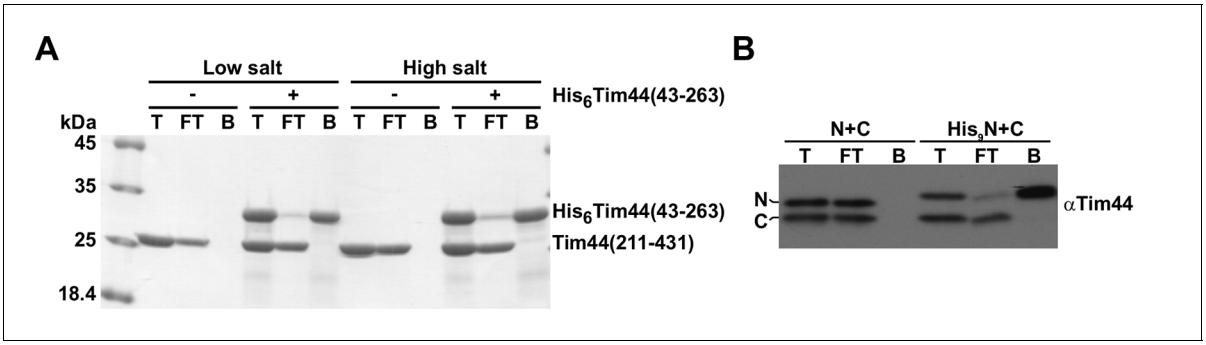

Figure 1—figure supplement 1. Two domains of Tim44 do not interact stably with each other. (**A**) Purified His_6-Tim44(43–263) was incubated with purified Tim44(211–431) either in low-salt (20 mM Tris/HCl, 50 mM NaCl, 10 mM imidazole, pH 8.0) or high-salt buffer (20 mM Tris/HCl, 300 mM NaCl, 10 mM imidazole, pH 8.0) for 5 min at 25˚C. The NiNTA-agarose beads were added and the mixture gently rolled for 30 min at 4˚C. After three washing steps with the same buffer, bound proteins were eluted with the buffer containing 300 mM imidazole. Total (T, 10%), flow-through (FT, 10%), and bound (B, 100%) fractions were analyzed by SDS–PAGE followed by Coomassie staining. (**B**) Mitochondria were isolated from yeast cells in which the function of the full-length Tim44 was rescued by coexpression of N- and C-terminal domains separately (N+C). In His_9N+C mitochondria, the N-terminal domain contained an additional His_9 tag. Mitochondria were solubilized with digitonin-containing buffer and incubated with NiNTA-agarose beads at 4˚C. After three washing steps, proteins specifically bound to the beads were eluted with Laemmli buffer containing 300 mM imidazole. Total (T, 10%), flow-through (FT, 10%), and bound (B, 100%) fractions were analyzed by SDS–PAGE followed by imunoblotting using antibodies to Tim44.
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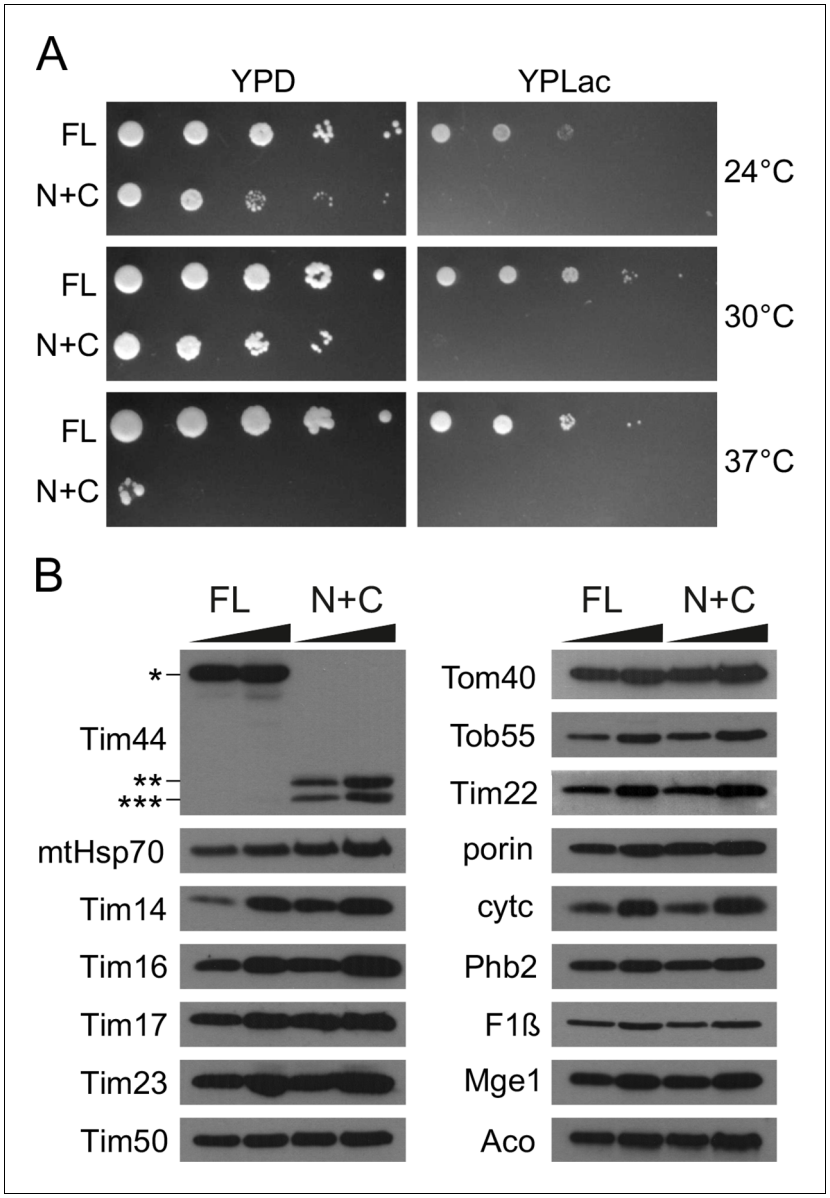

Figure 2. N+C cells grow poorly, even on fermentable carbon source. (**A**) Ten-fold serial dilutions of △*tim44* cells rescued by the wild-type, full-length copy of Tim44 (FL) or by its two domains expressed *in trans* (N+C) were spotted on rich medium containing glucose (YPD) or lactate (YPLac), as fermentable and non-fermentable carbon sources, respectively. Plates were incubated at indicated temperatures for 2 (YPD) or 3 days (YPLac). (**B**) 15 and 35 µg of mitochondria isolated from FL and N+C cells were analyzed by SDS–PAGE, followed by immunoblotting against depicted mitochondrial proteins.

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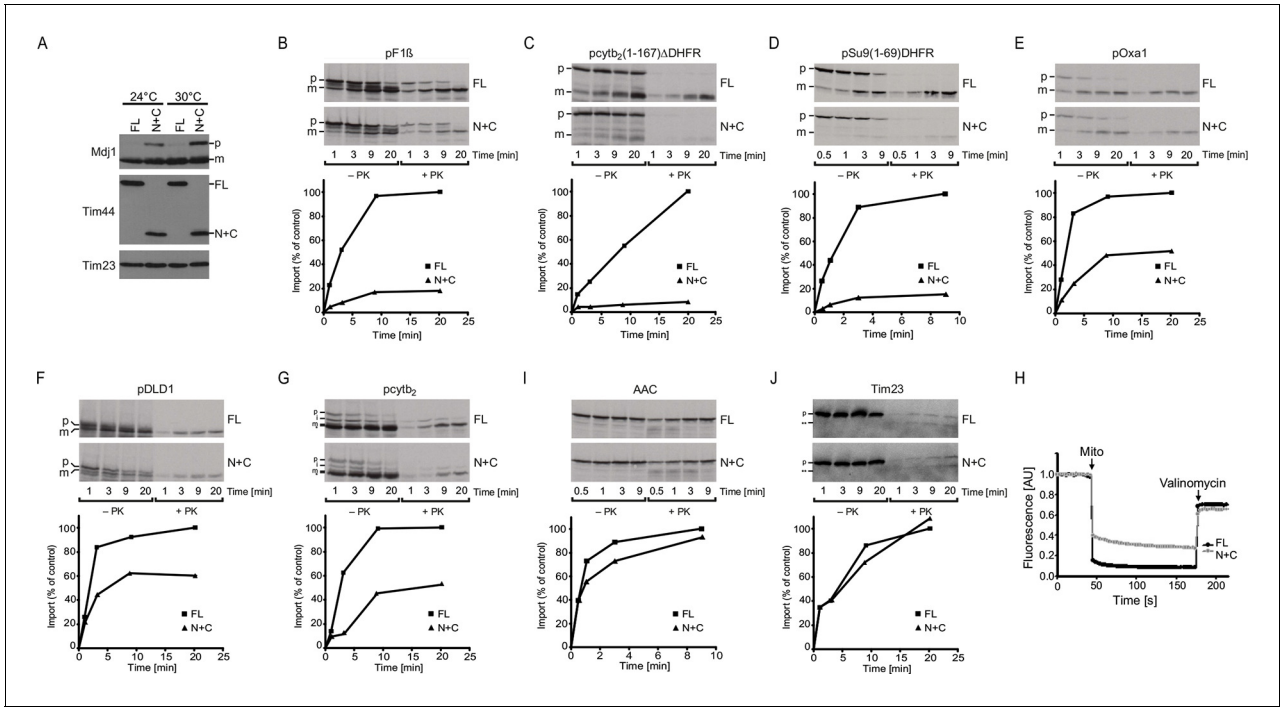

Figure 3. N+C cells have a strongly impaired import via the TIM23 complex. (**A**) Total cell extracts of FL and N+C cells grown at 24˚C and 30˚C were analyzed by SDS–PAGE and immunoblotting using indicated antibodies. p - precursor, and m - mature form of Mdj1. (**B–G** and **I–J**) ^{35}S-labeled mitochondrial precursor proteins were imported into mitochondria isolated from FL and N+C cells. After indicated time periods, aliquots were removed and Proteinase K (PK) was added where indicated. Samples were analyzed by SDS–PAGE, autoradiography and quantification of PK-protected mature forms of imported proteins. pF1β - precursor of the β subunit of F_oF_1 ATPase. pcytb_2(1–167)△DHFR - precursor consisting of the first 167 residues with the deleted sorting signal of yeast cytochrome b_2 fused to mouse dihydrofolate reductase (DHFR); pSu9(1–69)DHFR - matrix targeting signal (residues 1–69) of subunit 9 of F_oF_1 ATPase from *Neurospora crassa* fused to DHFR; pOxa1 - precursor of Oxa1; pDLD1 - precursor of D-lactate dehydrogenase; pcytb_2 - precursor of cytochrome b_2; AAC - precursor of ATP/ADP carrier; p, i, m - precursor, intermediate, and mature forms of imported proteins; * - *in vitro* translation product starting from an internal methionine. ** - clipped form of Tim23. (**H**) Membrane potential of isolated mitochondria was measured using $DiSC_3(5)$. Valinomycin was added to dissipate membrane potential.

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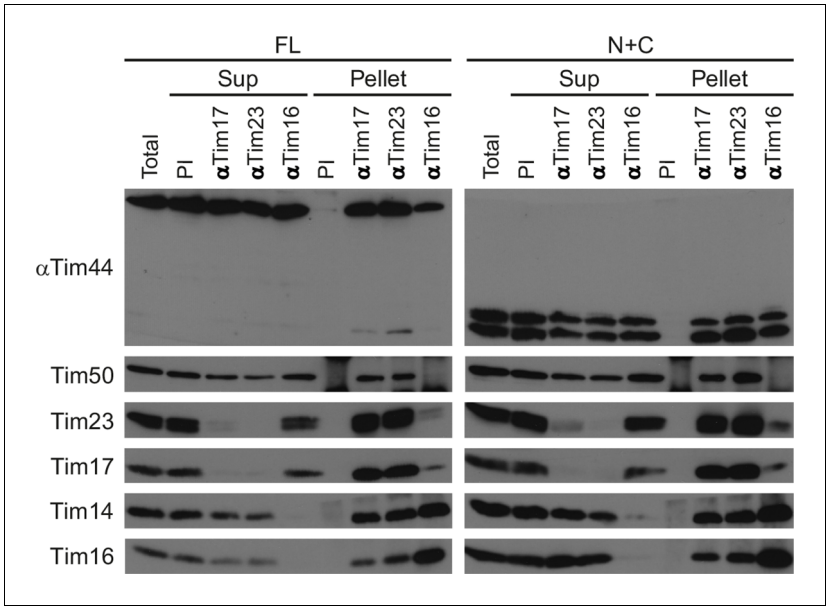

Figure 4. The TIM23 complex is assembled in N+C mitochondria. Mitochondria from FL and N+C cells were solubilized with digitonin-containing buffer and mitochondrial lysates incubated with affinity-purified antibodies to Tim17, Tim23, and Tim16 prebound to Protein A-Sepharose beads. Antibodies from preimmune serum (PI) were used as a negative control. After three washing steps, material specifically bound to the beads was eluted with Laemmli buffer. Total (20%), supernatant (Sup, 20%), and bound (Pellet, 100%) fractions were analyzed by SDS–PAGE and immunoblotting with indicated antibodies.

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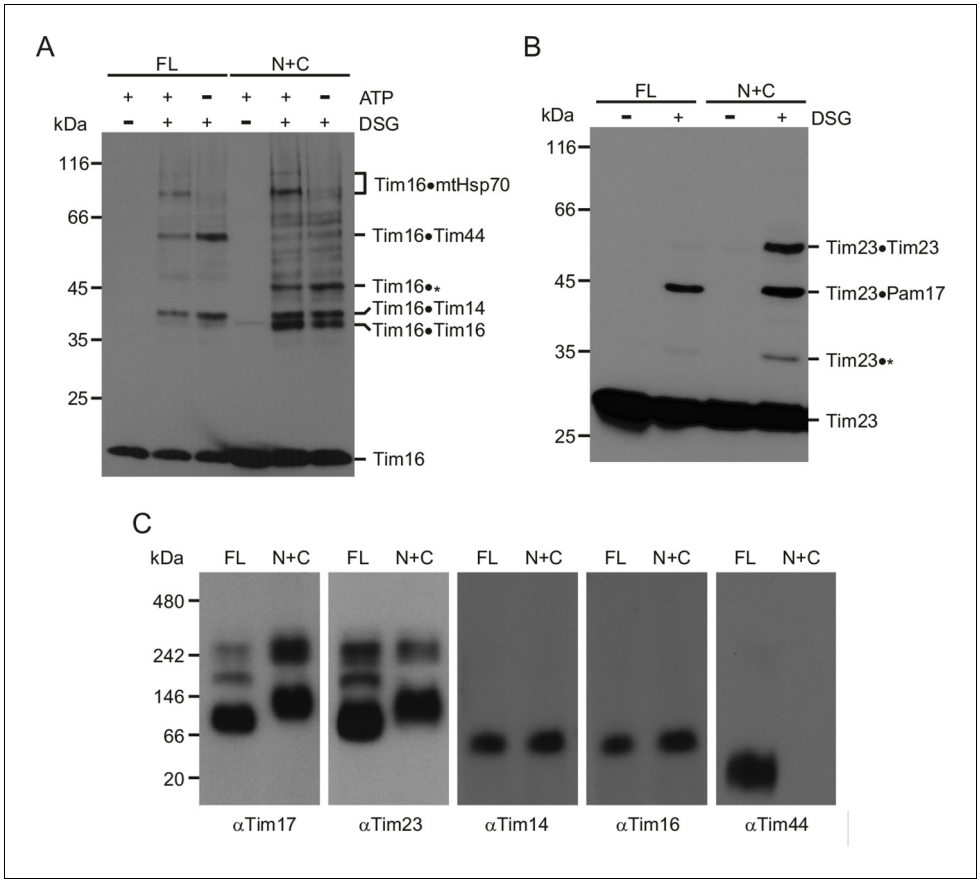

Figure 5. The TIM23 complex adopts an altered conformation in N+C mitochondria. (**A** and **B**) Mitochondria from FL and N+C cells were incubated with amino group-specific crosslinker disuccinimidyl glutarate (DSG). Where indicated, mitochondrial ATP levels were altered prior to crosslinking. After quenching of excess crosslinker, mitochondria were reisolated and analyzed by SDS–PAGE followed by immunoblotting with antibodies to Tim16 (**A**) and Tim23 (**B**). * indicates currently uncharacterized crosslinks. (**C**) Mitochondria from FL and N+C cells were solubilized in digitonin-containing buffer and analyzed by BN-PAGE and immunoblotting with indicated antibodies.

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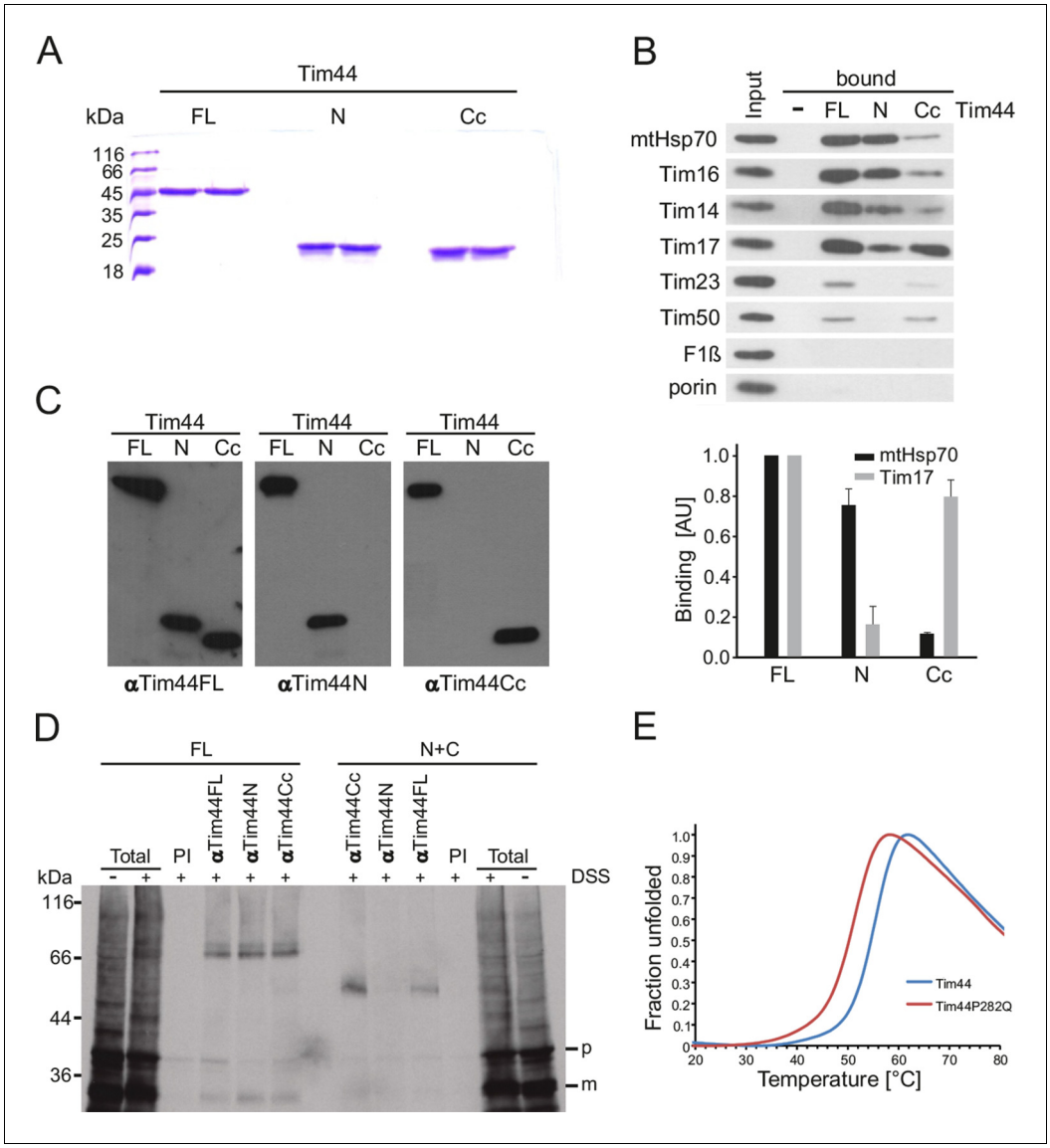

Figure 6. C-terminal domain of Tim44 interacts with Tim17 and with a precursor in transit. (**A**) Coomassie-stained SDS-PA gel of recombinantly expressed and purified constructs of Tim44. FL - full-length, mature Tim44 (residues 43–431); N - a construct encompassing the N-terminal domain of Tim44 (residues 43–209); Cc - a construct encompassing the core of the C-terminal domain of Tim44 (residues 264–431). (**B**) Wild-type mitochondria were solubilized with Triton X-100 and incubated with indicated purified constructs of Tim44 covalently coupled to CNBr-Sepharose beads. Beads with no coupled protein were used as a negative control. After washing steps, proteins specifically bound to the beads were eluted by Laemmli buffer and analyzed by SDS–PAGE followed by immunoblotting with the indicated antibodies. Input lane contains 4.5% of the material used for binding (upper panel). Binding of mtHsp70, as a representative of the import motor components, and of Tim17 to different beads was quantified from three independent experiments (lower panel). Binding to FL was set to 1. (**C**) Antibodies specific for N and Cc domains of Tim44 were affinity purified from rabbit serum raised against full-length Tim44 using respective domains of Tim44 covalently coupled to Sepharose beads, as described under (**B**). To test the specificity of purified antibodies, indicated Tim44 constructs were loaded on an SDS-PA gel, blotted on a nitrocellulose membrane and obtained membranes were immunoblotted using the purified antibodies, as indicated. (**D**) ^{35}S-labelled matrix targeted precursor protein pcytb_2(1–167)ΔDHFR was imported into isolated mitochondria from FL and N+C cells in the presence of methotrexate, leading to its arrest as a TOM-TIM23 spanning intermediate. Samples were then crosslinked with disuccinimidyl suberate (DSS), where indicated. After quenching of excess crosslinker, aliquots were taken out for 'total' and the rest of samples solubilized in SDS-containing buffer to dissociate all noncovalent protein–protein interactions. Solubilized material was incubated

Figure 6 continued on next page

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with indicated affinity-purified antibodies prebound to Protein A-Sepharose beads. Antibodies from preimmune serum (PI) were used as a negative control. Material specifically bound to the beads was eluted with Laemmli buffer and analyzed by SDS–PAGE and autoradiography. p - precursor and m - mature forms of pcytb_2(1–167)Δ DHFR. (**E**) Melting curves of recombinant wild type and Pro282Gln mutant of Tim44 obtained by thermal shift assay.

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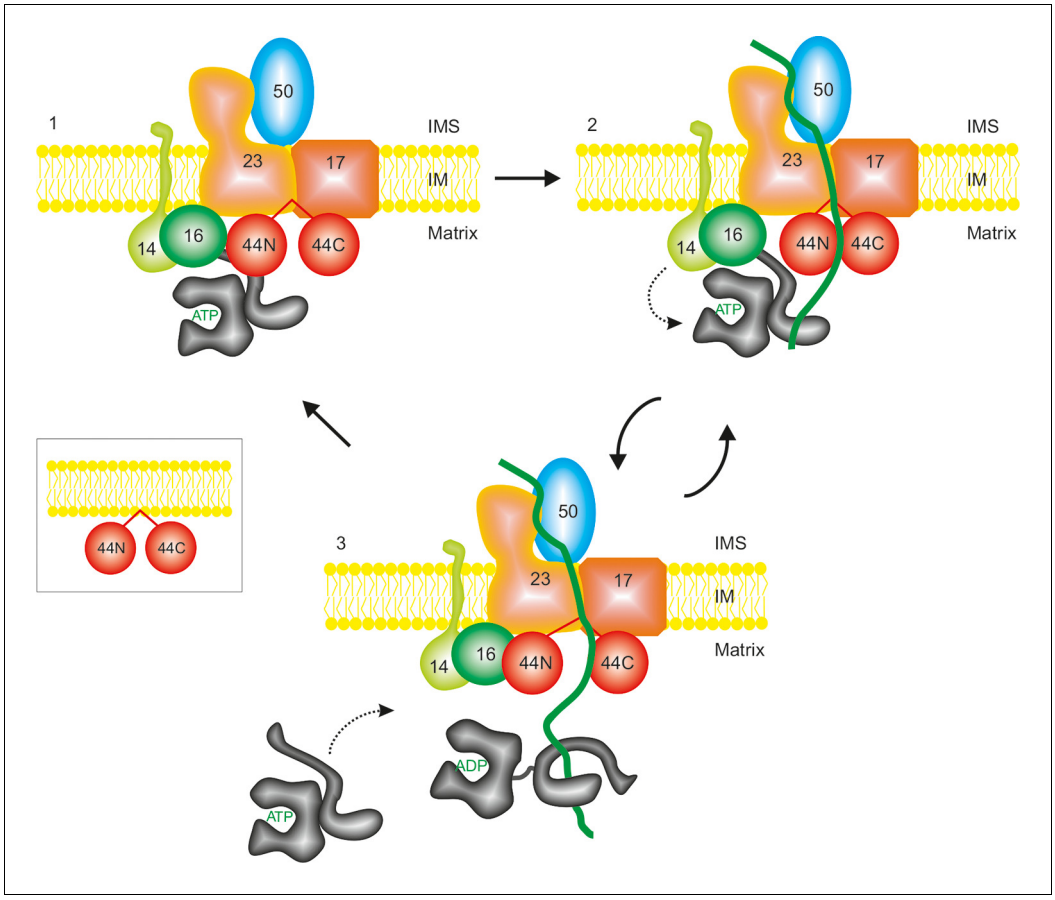

Figure 7. A proposed model of function of the TIM23 complex. See text for details. For simplicity reasons, only essential subunits of the complex are shown.
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