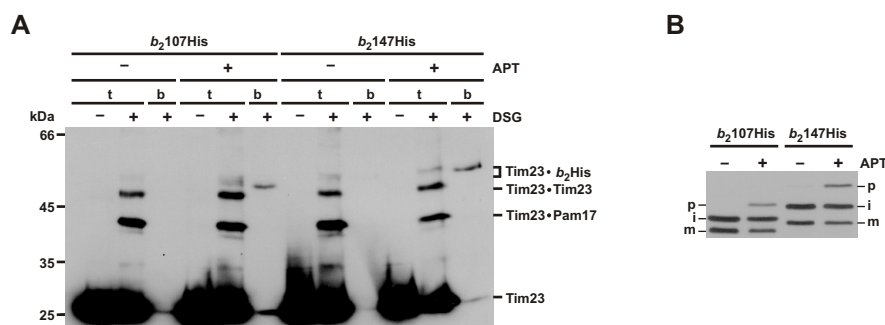
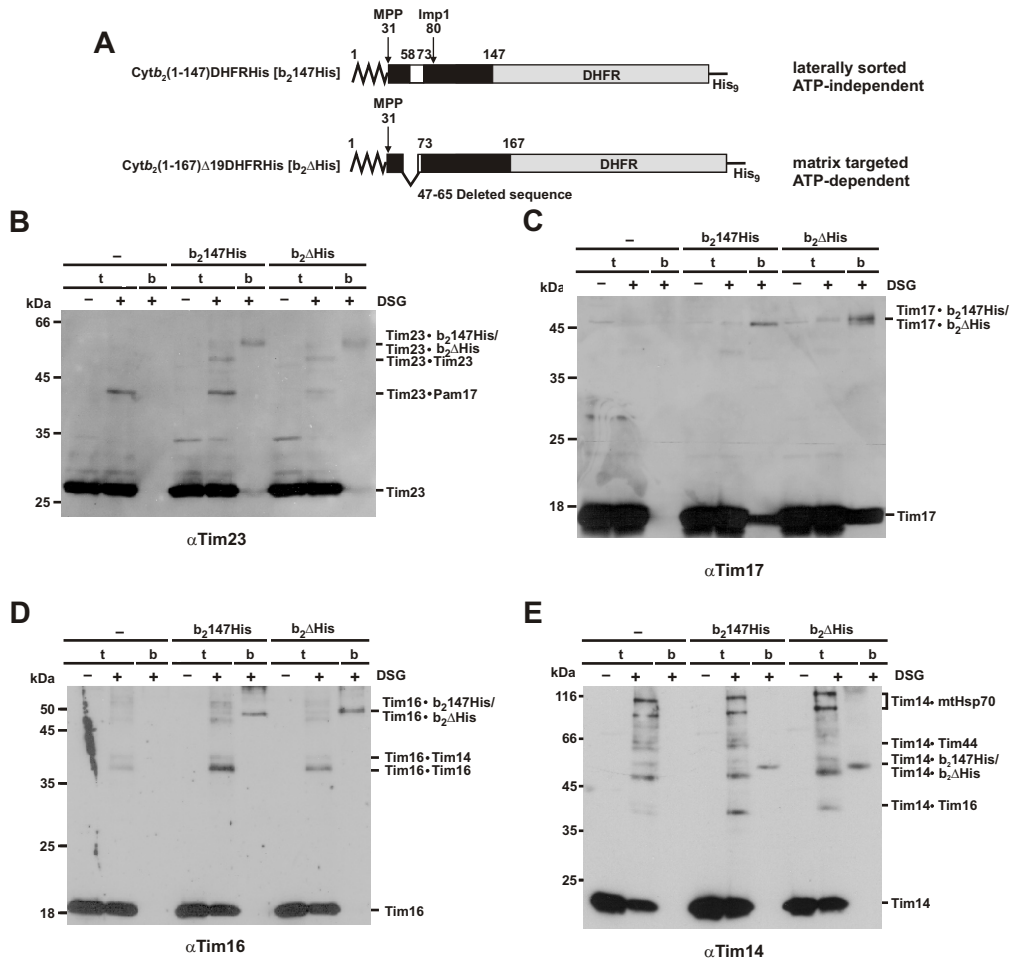


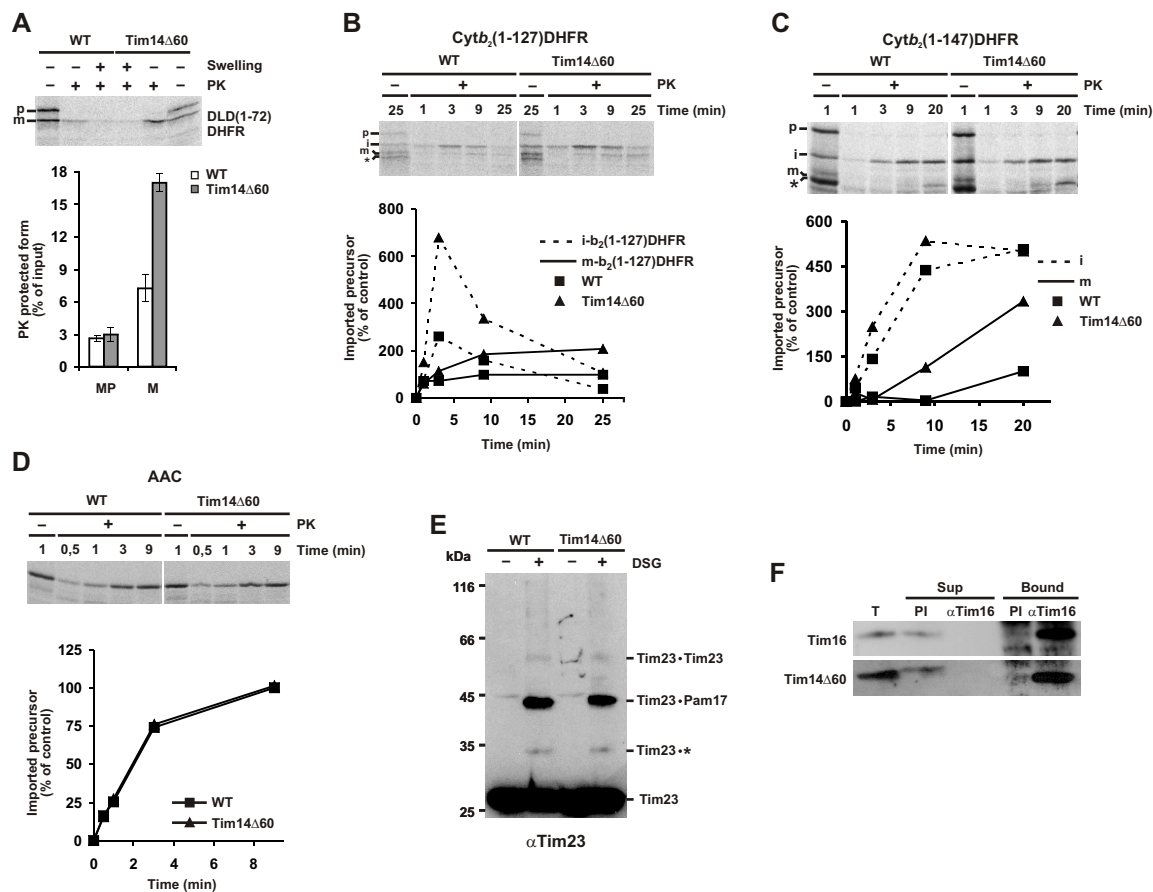
**Supplementary Figure 1. Translocation arrest of substrates laterally sorted by the TIM23 complex.** Schematic representation of fusion proteins consisting of different segments of yeast cytochrome b2 (Cytb2) (black box) and mouse dihydrofolate reductase (DHFR) (grey box). Zigzag line, presequence; white box, transmembrane segment of b2; MPP, mitochondrial processing peptidase; IMP, inner membrane protease; HBD, haem-binding domain. (B) <sup>35</sup>S-labeled cytb2(1-167)DHFR was imported into wild type mitochondria in the presence or absence of DHF and NADPH. DHF/NADPH-treated samples were reisolated, washed and incubated further for 5 and 20 min (chase). Samples were treated with Proteinase K (PK) where indicated and analyzed by SDS-PAGE and autoradiography. p, precursor, i, intermediate, and m, mature forms of imported protein. \*, a translation product arising from an internal methionine (C-E) Wild type and *ssc1-3* mitochondria were preincubated for 10 min at 37°C and used for import of indicated precursor proteins in the presence of DHF and NADPH. Mitochondria were reisolated, incubated further without NADPH/DHF for indicated time periods and subsequently treated as described under (B). Autoradiographs are shown in upper panels and quantifications of PK-protected material in the lower ones. PK-protected mature forms at 30 min in WT were set to 100%.



**Supplementary Figure 2. Dependence of crosslinking to Tim23 on the translocation arrest of laterally sorted precursors.** Chimeras consisting of 107 and 147 residues of cytochrome b2 and full length mouse dihydrofolate reductase (DHFR) with the C-terminal His tag, b2107His and b2147His, were expressed in yeast cells in the presence or absence of aminopterin (APT). Mitochondria were isolated and subjected to crosslinking with disuccinimidylglutarate (DSG). Samples were either directly resuspended in Laemmli buffer or were first solubilized in SDS-containing buffer and incubated with NiNTA-Agarose beads to isolate His-tagged chimeras and their crosslinking adducts. All samples were analyzed by SDS-PAGE followed by immunodecoration with antibodies to Tim23. t, total t (10%); b, bound fraction (100%). Known crosslinking adducts are indicated. (B) Isolated mitochondria were analyzed by SDS-PAGE and immunodecoration with antibodies to DHFR. P, precursor, i, intermediate and m, mature forms of chimeras.



**Supplementary Figure 3. Crosslinking of laterally sorted and matrix targeted precursors to the TIM23 subunits.** (A) Schematic representation of precursor proteins used. Note that the lengths of precursor forms of both chimeras are essentially identical. The same is true for the intermediate form of *cytb*<sub>2</sub>(1-147)DHFRHis and the mature form of *cytb*<sub>2</sub>(1-167)Δ19DHFRHis. (B-E) Indicated precursor proteins were expressed in yeast cells in the presence of aminopterin and isolated mitochondria were subjected to crosslinking with DGS. Part of the samples was immediately prepared for SDS-PAGE, t - totals (5%), and the rest was solubilized with SDS-containing buffer and incubated with NiNTA beads. Specifically bound material was eluted with Laemmli buffer containing 300 mM imidazole, b - bound (100%). Samples were analyzed by SDS-PAGE and immunodecoration with antibodies to Tim23 (B), Tim17 (C), Tim16 (D) and Tim14 (E).



**Supplementary Figure 4. Effects of deletion of the IMS domain of Tim14 on the structure and function of the TIM23 complex.** (A) Mitochondria were preincubated in import buffer for 10 min at 37°C. <sup>35</sup>S-labeled DLD(1-72)DHFR was added and samples were incubated further at 25°C for 12 min. Samples were subjected to hypotonic swelling and treated with proteinase K (PK) where indicated. Reactions were analyzed by SDS-PAGE and autoradiography (upper panel). Quantification of the mature form is shown in the lower panel. MP, mitoplasts; M, mitochondria. (B-D) Mitochondria were incubated for 10 min at 37°C prior to addition of indicated radiolabeled precursors and further incubation at 25°C. At the indicated time points samples were removed and treated with Proteinase K (PK) where indicated. Samples were analyzed by SDS-PAGE and autoradiography (upper panel). Quantification of the PK-protected mature form is shown in the lower panel. The signal obtained at the longest time point in wild type mitochondria was set to 100%. p, precursor, i, intermediate and m, mature form of the imported protein. (E) Mitochondria were incubated for 10 min at 37°C and then subjected to crosslinking with DSG. Samples were analyzed by SDS-PAGE and immunodecoration with antibodies against Tim23. (F) Tim14 $\Delta$ 60 mitochondria were incubated for 10 min at 37°C, reisolated and solubilized with Triton X-100. Solubilized material was incubated with affinity purified antibodies to Tim16 and antibodies from preimmune serum (PI) prebound to Protein A-Sepharose. Specifically bound material was eluted with Laemmli buffer. Total, t (20%), material remaining in the supernatant, sup (20%) and bound material (100%) were analyzed by SDS-PAGE and immunodecoration with antibodies to Tim16 and Tim14.