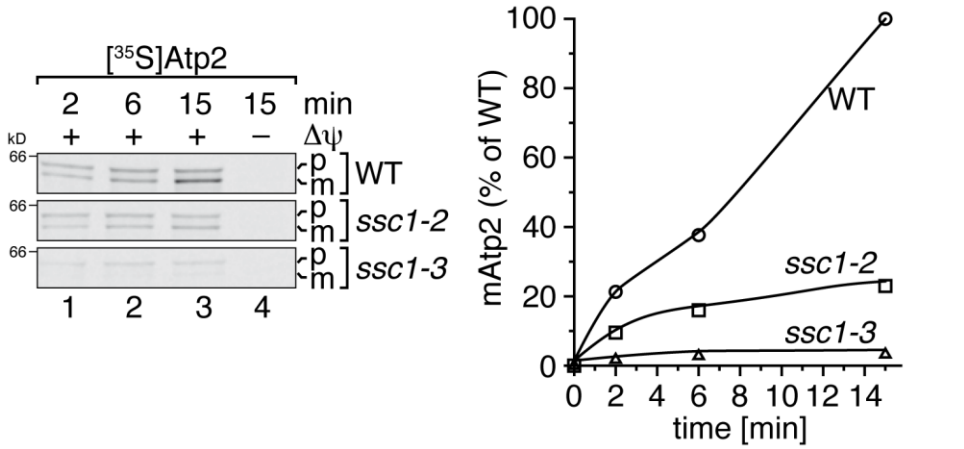
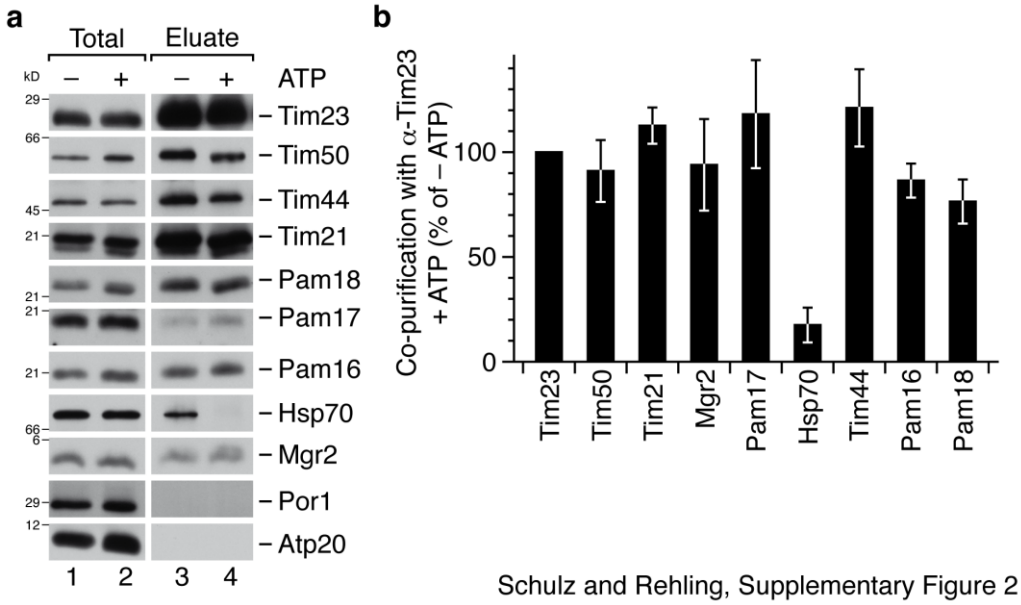
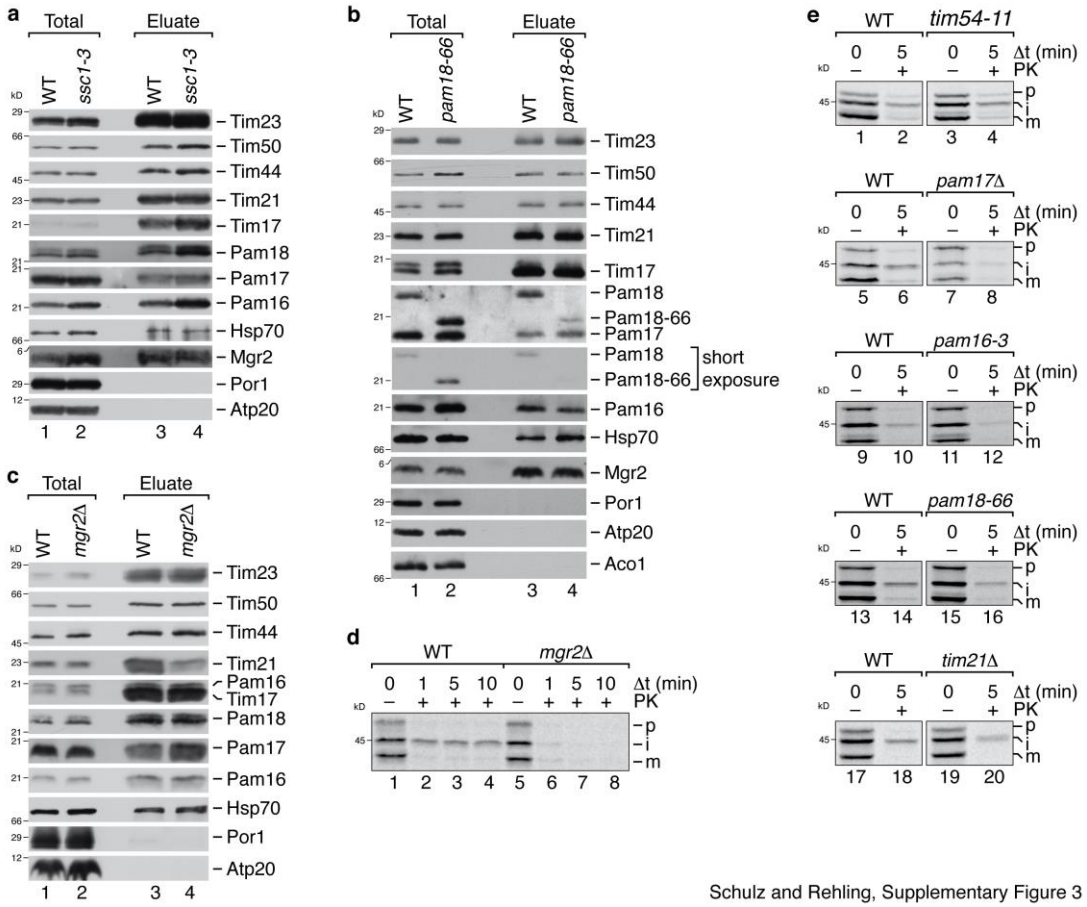


Schulz and Rehling, Supplementary Figure 1

Supplementary Figure 1: Import defects in *ssc1* temperature sensitive mutants. Isolated WT, *ssc1-2* and *ssc1-3* mitochondria were subjected to 15 min heat shock. Subsequently, [^{35}S]Atp2 was imported for the indicated times. The reaction was stopped by dissipation of $\Delta\psi$ and proteinase K treatment. Samples were analyzed by SDS-PAGE and digital autoradiography (left). Quantification of the amount of mature Atp2 is shown (right, 100 %: amount imported in WT after 15 min). p, precursor; m, mature.

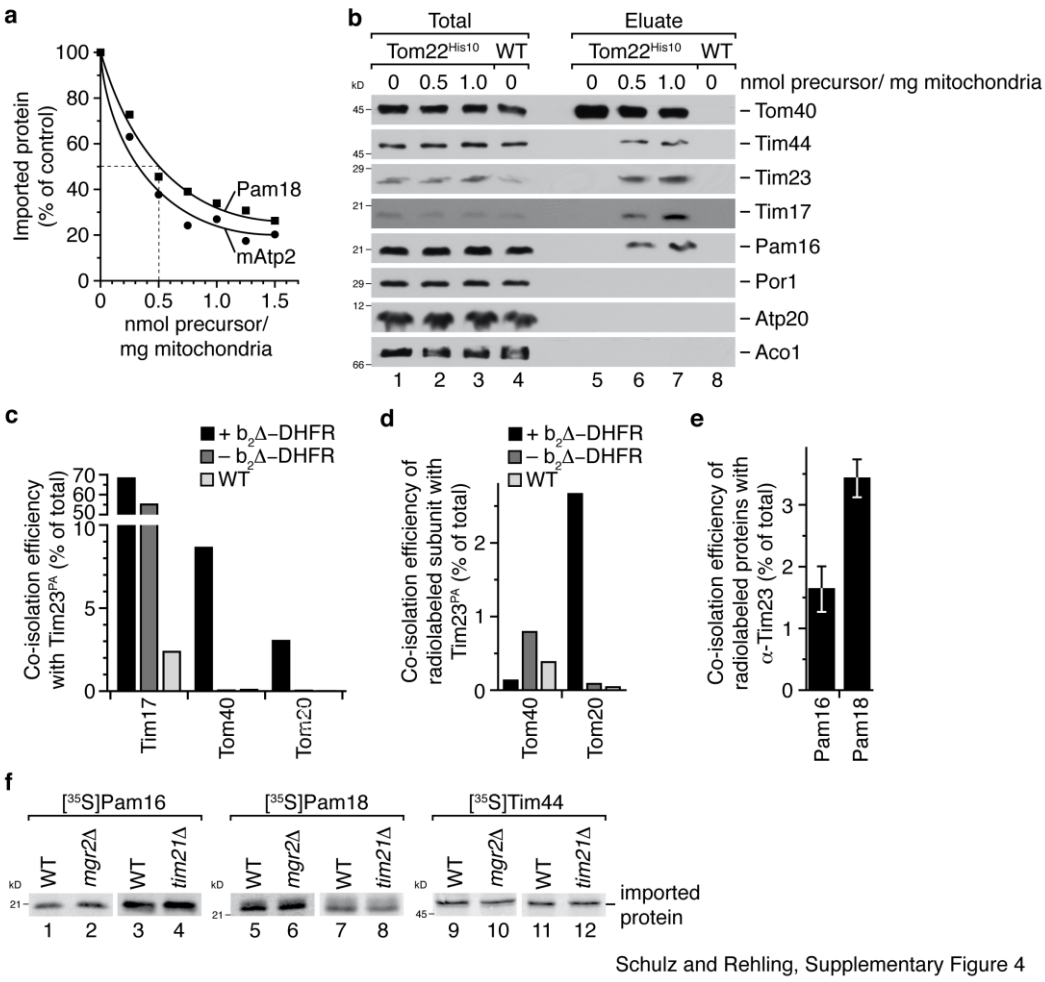


Supplementary Figure 2: Recovery of the TIM23 complex by α-Tim23 co-immunoprecipitations. (**a**) α-Tim23 co-immunoprecipitations were performed from WT mitochondria solubilised with digitonin in the absence or presence of ATP. Samples were analyzed by SDS-PAGE and Western-blotting. Total, 5%; eluate, 100%. (**b**) Quantification of experiments performed as described in (**a**). The co-purification with Tim23 and +ATP is shown as % of –ATP sample (normalized to Tim23, mean ± SEM, n=3).



Supplementary Figure 3: TIM23 complex composition in mutants and inward driving activity. (**a**) Isolated wild type (WT) and *ssc1-3* mitochondria were treated at 37°C prior to solubilisation with digitonin buffer and subjected to α-Tim23 co-immunoprecipitation. Samples were analyzed by SDS-PAGE and Western-blotting. Total, 5%; eluate, 100%. The co-isolation efficiency for Pam16 in mutant mitochondria was reproducibly similar to the wild type sample upon quantification. (**b**) Experiment as described in (**a)** performed with WT and *pam18-66* mitochondria. α-Pam18 efficiently recognizes the mutant form of Pam18 (lane 2). (**c**) Experiment performed as in (**a)** with WT and *mgr2*Δ mitochondria except that the heat-shock was omitted. (**d**) Inward driving activity using b_2(220)-DHFR as described in Fig. 1g. Samples were analyzed by SDS-PAGE and autoradiography. (**e**) Inward driving activity using

b_2(220)-DHFR as described in Fig. 1h. Samples were analyzed by SDS-PAGE and autoradiography.



Supplementary Figure 4: Determination of semi-saturating amounts of $b_2(167)\Delta$-DHFR for TIM23/TOM co-isolation efficiency. (**a**) The indicated amounts of $b_2(167)_\Delta$-DHFR were arrested in mitochondria using MTX for 15 min at 25°C. After reisolation, [^{35}S]Atp2 (circle) and [^{35}S]Pam18 (square) were imported for 30 min at 25°C and $\Delta\psi$ was dissipated. The reaction was proteinase K (PK) treated and analyzed by SDS-PAGE and digital autoradiography. The amount of imported protein is shown (100%, amount imported in the absence of arrested $b_2(167)_\Delta$-DHFR). (**b**) 0, 0.5 or 1.5 nmol $b_2(167)_\Delta$-DHFR per mg mitochondria were arrested using MTX for 15 min at 25°C. After $\Delta\psi$ dissipation the reaction was chased for 5 min at 25°C and washed. Samples were solubilised with digitonin and Tom22^{His10} was isolated.

Analysis by SDS-PAGE and Western-blotting. Total, 5%; eluate, 100%. (**c**) Quantification of experiments shown in Figure 3b and c. The co-isolation efficiency of the authentic subunits is shown for the isolation with $Tim23^{PA}$ arrested with $b_2(167)_\Delta$-DHFR (black bar), without $b_2(167)_\Delta$-DHFR (dark gray bar) or in wild type (WT) without $b_2(167)_\Delta$-DHFR (light gray bar) (% of total). (**d**) Quantification of experiments shown in Figure 3b and c. The co-isolation efficiency of the radiolabeled subunits with $Tim23^{PA}$ is shown (% of total). Bars as in (**c**). (**e**) Radiolabeled Pam16 and Pam18 were imported into WT mitochondria for 20 min. After dissipation of the $\Delta\psi$ a part of the reaction was proteinase K treated (imported fraction), the remainder was solubilized and subjected to α-Tim23 co-immunoprecipitation. Samples were analyzed by SDS-PAGE, autoradiography and quantification. The co-isolation efficiency is shown as % of imported (n=3, SEM). (**f**) Radiolabeled Pam16, Pam18 and Tim44 were imported into *mgr2*Δ or *tim21*Δ mitochondria (and the respective WT) for 20 min to saturation of the import reaction. After dissipation of the $\Delta\psi$ a part of the reaction was proteinase K treated and analyzed by SDS-PAGE and autoradiography. The remaining reaction was processed as described in Fig. 4b.

Supplementary Figure 5

Original scans of key Western blots and gels presented in the paper

Figure 1d

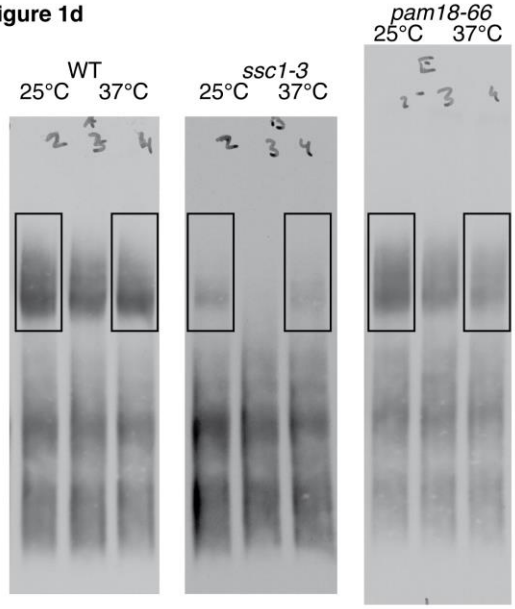
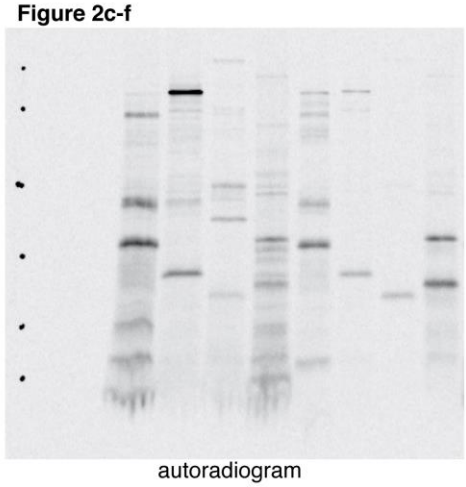
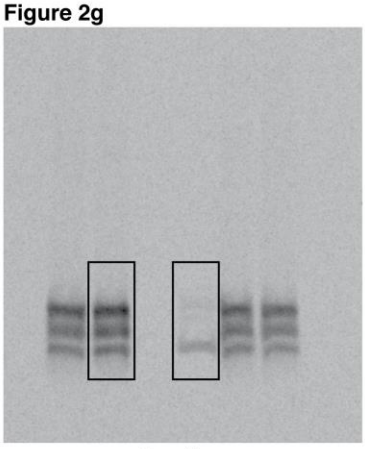


Figure 2c-f



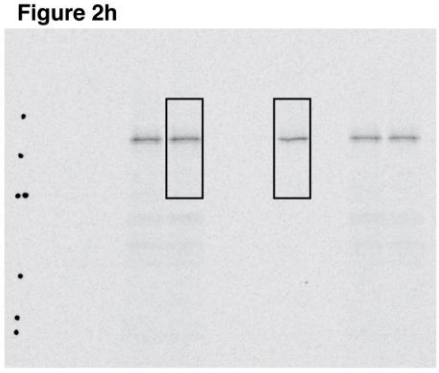
autoradiogram

Figure 2g



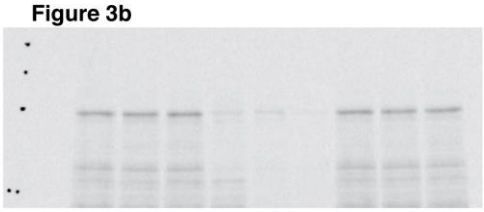
autoradiogram

Figure 2h



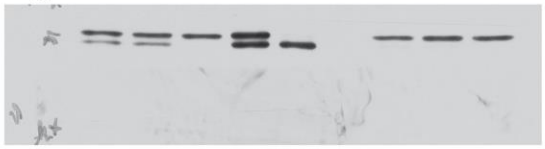
autoradiogram

Figure 3b



autoradiogram

Figure 3b

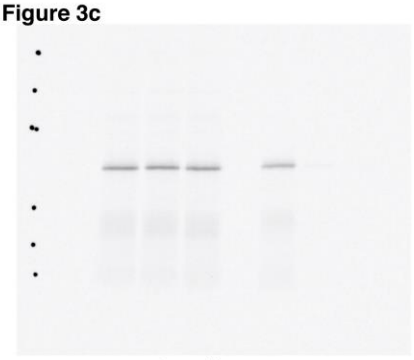


IB: Tom40

IB: Tim21

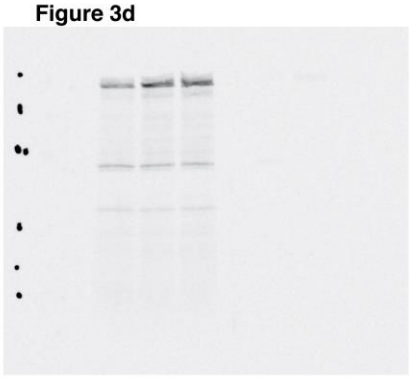
Schulz and Rehling, Supplementary Figure 5

Figure 3c



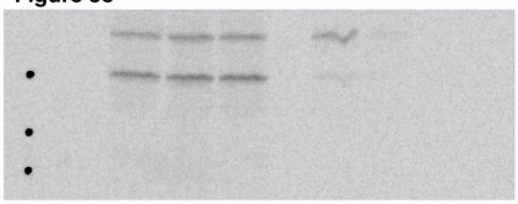
autoradiogram

Figure 3d



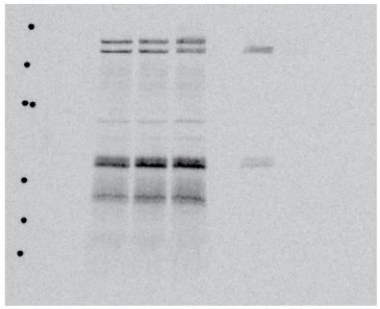
autoradiogram

Figure 3e



autoradiogram

Figure 3f



autoradiogram

Figure 3c

IB: Tom20

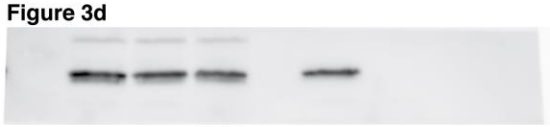
Figure 3c

IB: Tim17

Figure 3d

IB: Tom40

Figure 3d



IB: Tim23

Figure 3e

IB: Tom40

Figure 3e

IB: Tim21/ Pam16

Figure 3f

IB: Tim44

Figure 3f

IB: Pam18

Figure 3f

IB: Tom40

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