Schendzielorz et al., https://doi.org/10.1083/jcb.201607066

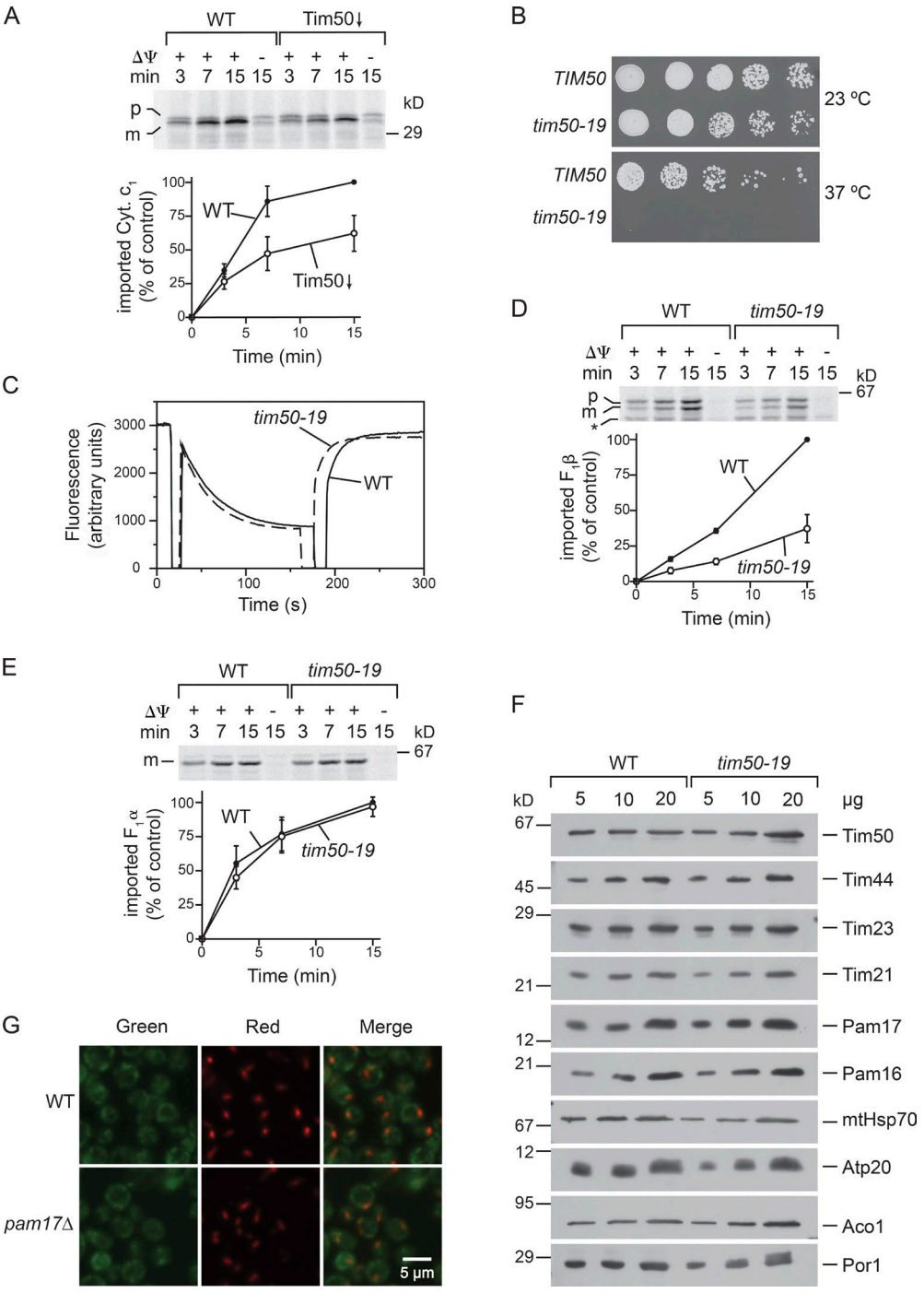

Figure S1. **Corresponding import experiments to Fig. 1 in *tim50-19* mitochondria.** (A) ^{35}S-labeled precursor was imported as described in Fig. 1 for the indicated times. After stopping the import reaction with AVO, all samples were PK treated and analyzed by SDS-PAGE and digital autoradiography. (B) Serial dilutions of WT and *tim50-19* cells were spotted onto YPD medium and incubated at 23°C or 37°C. (C) Membrane potential of WT and *tim50-19* mitochondria was assessed as described in Figs. 1 and 2. (D and E) Isolated mitochondria were incubated for 15 min at a nonpermissive temperature before import of ^{35}S-labeled precursors for the indicated times. After stopping import, all samples were PK treated and analyzed by SDS-PAGE and digital autoradiography. The asterisk indicates an unspecific degradation product. (A, D, and E) Results are presented as mean ± SEM. $n = 3$. p, precursor; m, mature protein. (F) Steady-state Western blot analysis of mitochondria isolated from WT and *tim50-19*. (G) WT and *pam17Δ* cells were grown in YPG medium at 25°C overnight. To analyze mitochondrial membrane potential, cells were harvested and incubated with 20 µM CCCP or the same volume of ethanol for 5 min at 25°C before the addition of 2 µM JC-1 for 10 min at 25°C. JC-1 accumulates in a membrane potential–dependent manner in mitochondria and changes its emission spectrum from green to red light.

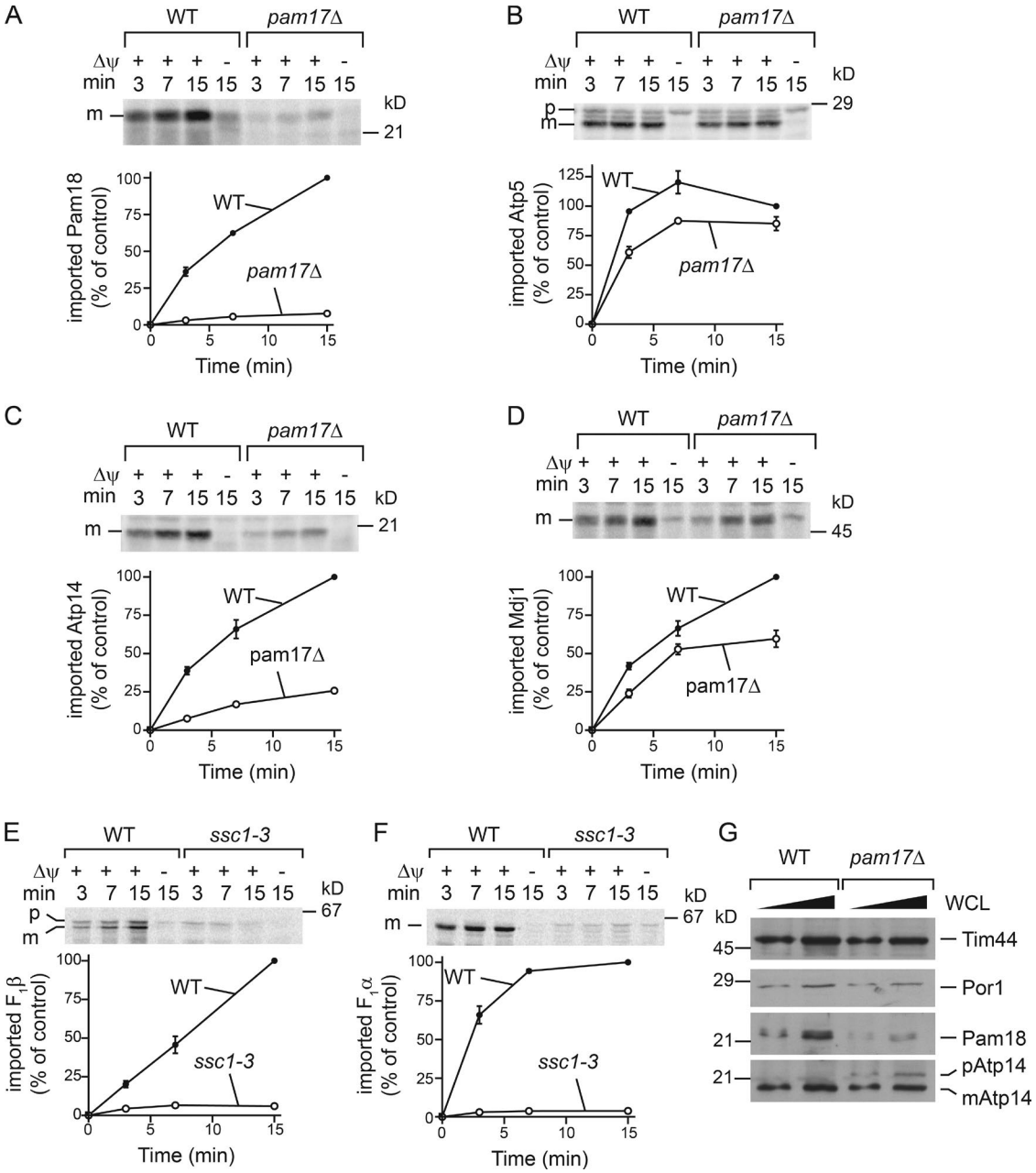

Figure S2. **Protein import is impaired in *pam17Δ* mitochondria and cells.** (A–D) ^{35}S-labeled precursors were imported into isolated mitochondria as described in Fig. 1. After 15 min of import, reactions were stopped and import was analyzed by SDS-PAGE and autoradiography. Results are presented as mean ± SEM. *n* = 3. (E and F) Mitochondria were incubated at 37°C for 15 min, and import was performed as described in A–D. p, precursor; m, mature protein. (G) To assess mitochondrial precursor accumulation in cells, WT and *pam17Δ* cells were grown overnight in YPD medium at 30°C. Cells were diluted in YPG medium and grown for 10 h. Subsequently, cells were harvested and cell lysates were analyzed by SDS-PAGE and Western blotting. WCL, whole cell lysate amount.

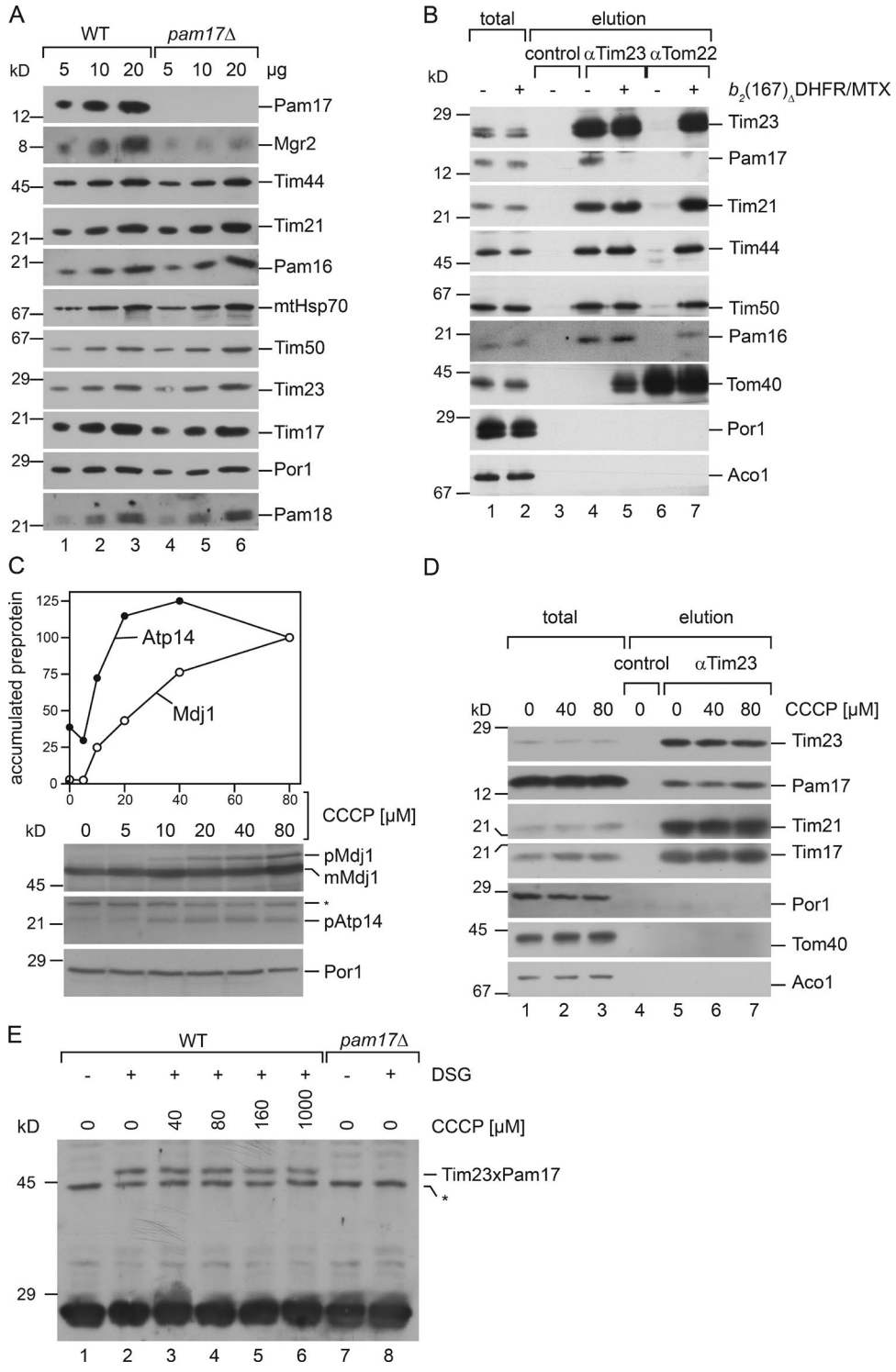

Figure S3. **Pam17 is not part of the active motor complex, and its association with TIM23 complex is not Δψ dependent.** (A) Steady-state Western blot analysis of mitochondria isolated from WT and *pam17Δ*. (B) Mitochondria were incubated in import buffer with or without b_2(167)Δ-DHFR and MTX for 15 min at 25°C. Afterward, mitochondria were solubilized in digitonin, and TIM23 and TOM complexes were immunoisolated using Tim23- and Tom22-specific antibodies, respectively. Total (5%) and elution fractions were analyzed by SDS-PAGE and Western blotting. (C) Cells were grown overnight at 30°C. After dilution, cells were grown for an additional 5 h in 2× YPAD medium at 30°C. Cell amounts were adjusted to OD_{600} = 1, and indicated amounts of CCCP were added for 30 min at 30°C. Cells were harvested and lysed before analysis by Western blotting. The amounts of Mdj1- and Atp14-accumulated precursors were quantified and plotted as percentages of accumulated precursors at 80 µM CCCP. The asterisk indicates a nonspecific cross-reaction band. *n* = 1. (D) Mitochondria were resuspended in solubilization buffer without digitonin and treated with the indicated amounts of CCCP for 5 min on ice. Afterward, digitonin was added and the TIM23 complex was isolated using Tim23-specific antibodies. Total (10%) and elution fractions were analyzed by SDS-PAGE and Western blotting. (E) Isolated mitochondria were incubated with indicated amounts of CCCP for 5 min before cross-linking with the amino group–specific cross-linker disuccinimidyl glutarate (DSG). After quenching of the cross-linker with glycine, mitochondria were reisolated and analyzed by SDS-PAGE and Western blotting. The asterisk indicates the nonspecific signal of the antibody.