

Expanded View Figures

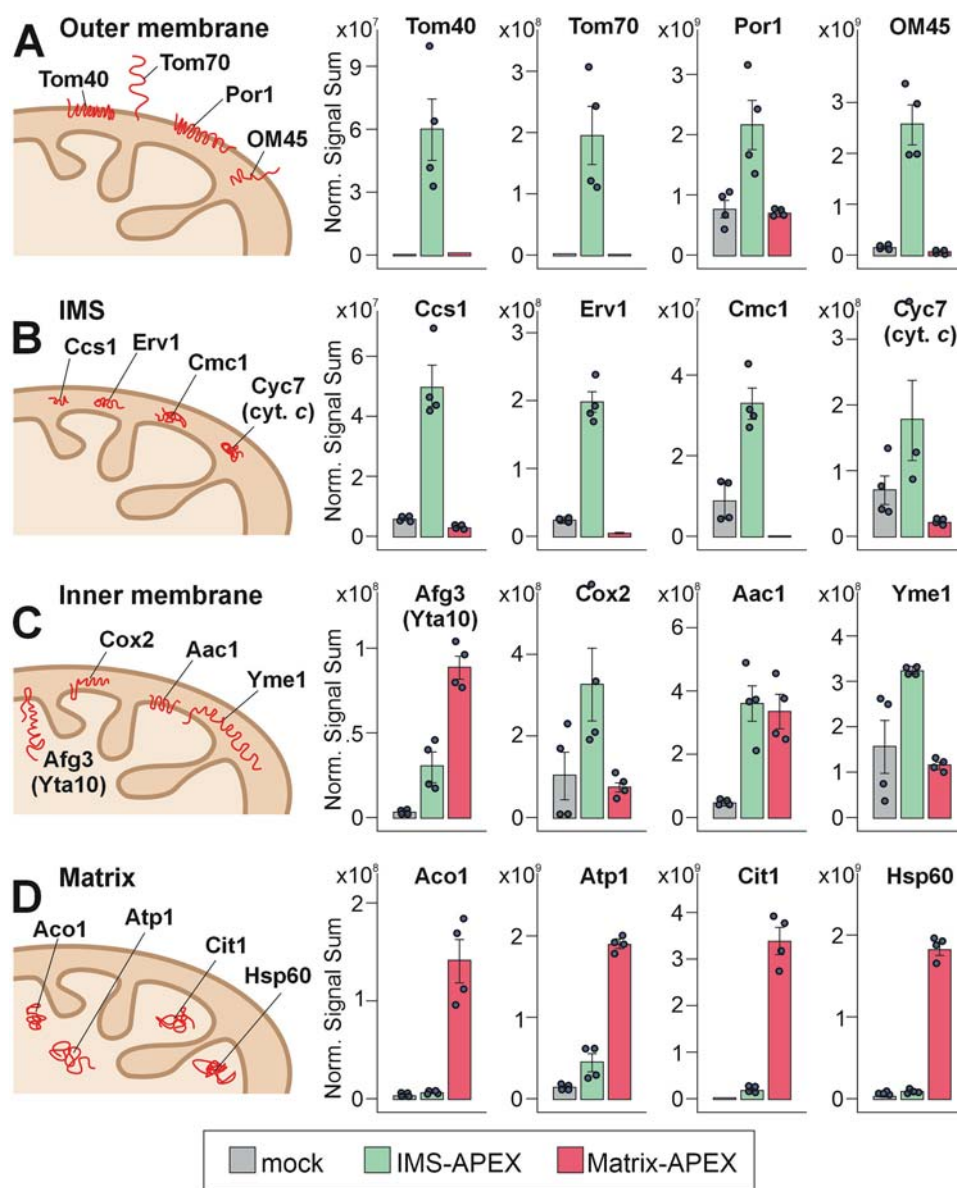
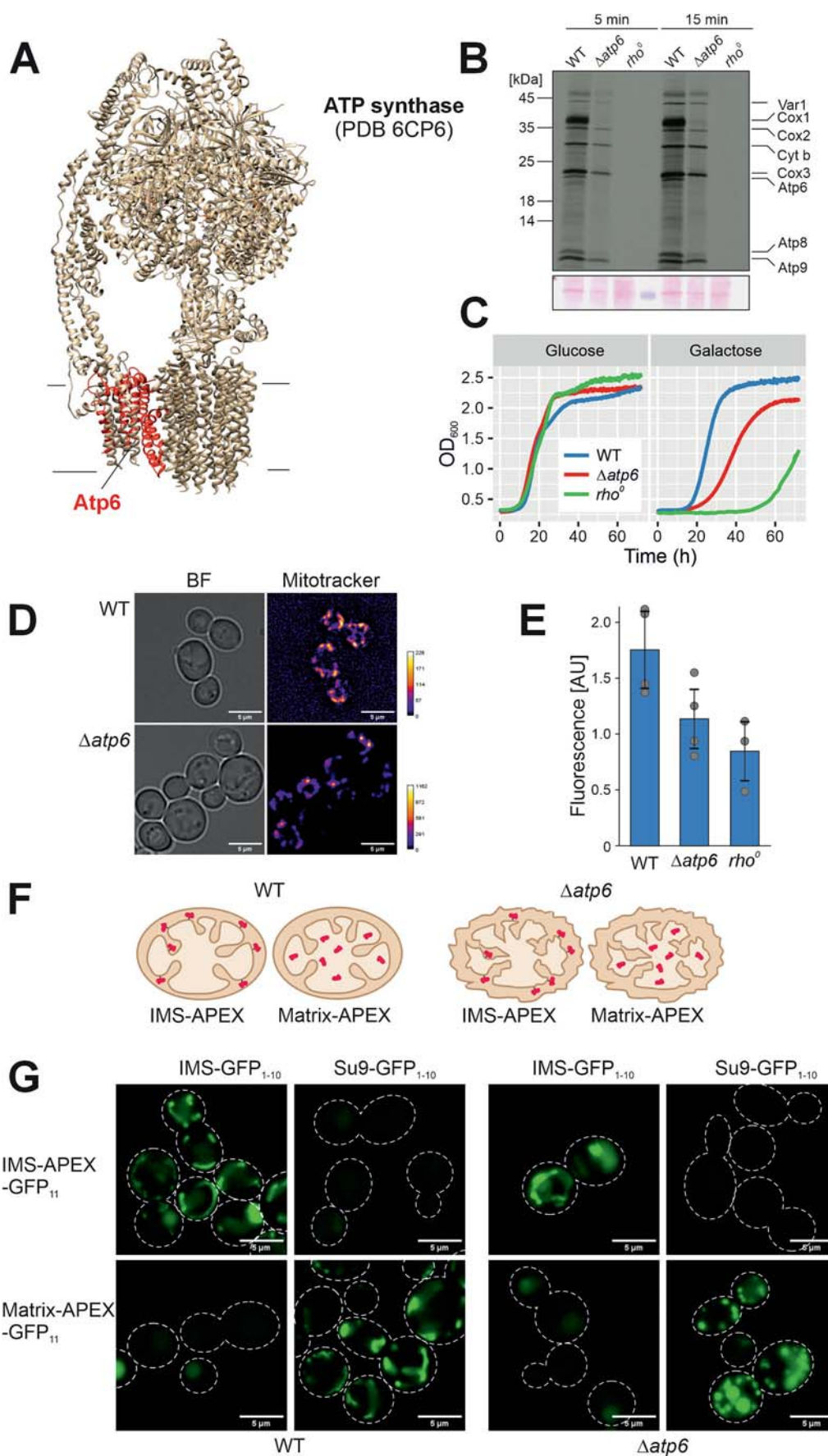


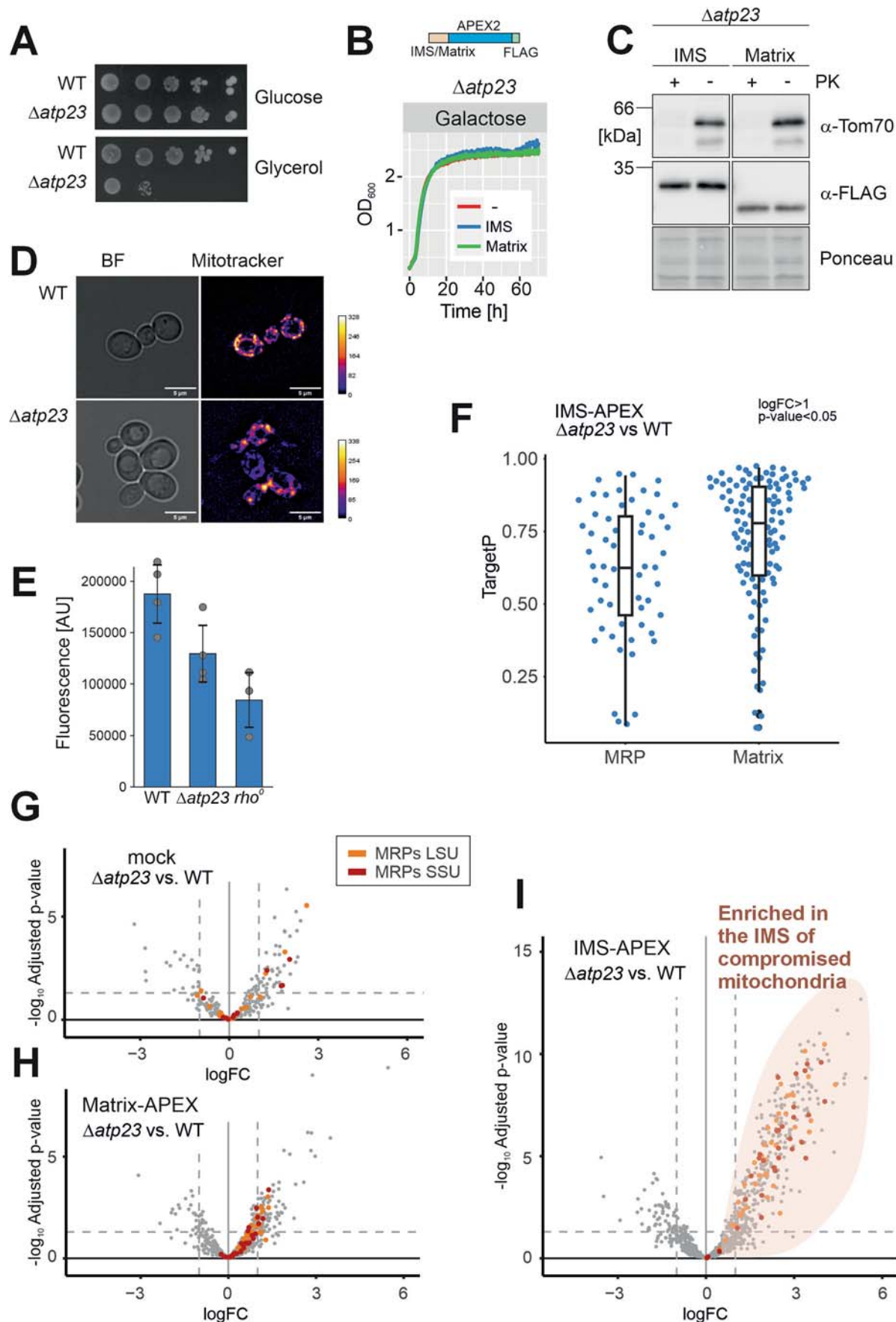
Figure EV1. APEX-mediated biotinylation patterns reveal intramitochondrial distribution of proteins.

(A-D) Protein levels were detected by mass spectrometry in the streptavidin pulldowns from lysed isolated mitochondria, derived from cells expressing no APEX (gray), or IMS-APEX (green) or matrix-APEX (red). Shown are the normalized signal sums of mitochondrial proteins of the four different mitochondrial subcompartments. Plotted were mean values and standard deviations from four replicates. The intramitochondrial localizations of the proteins are sketched on the left. See Dataset EV1 for details.



◀ **Figure EV2. A mutant lacking the mitochondrially encoded *ATP6* gene serves as example for mitochondrial dysfunction.**

(A) Structure of the ATPase complex of baker's yeast (Srivastava et al, 2018). Atp6 (shown in red) is a subunit of the F_o part that plays a crucial role in proton pumping. (B) Mitochondria were isolated from the indicated strains and incubated in *in organello* translation buffer in the presence of ^{35}S -methionine for 5 or 15 min. Radiolabeled translation products were separated by SDS-PAGE and visualized by autoradiography. (C) Growth of the indicated strains in synthetic medium containing the indicated carbon sources was recorded continuously and plotted. Shown are mean values of three technical replicates. (D) Wild-type and Δatp6 cells were grown in galactose medium. Mitochondria were stained with the membrane potential-dependent dye mitotracker CMXRos. Intensity values were calculated to indicate the degree of mitochondrial energization in the two strains. (E) The fluorescence intensities of the mitotracker-stained mitochondria were quantified as a measure of the mitochondrial membrane potential in the strains. Shown are mean values and standard deviations from four biological replicates. (F) Schematic representation of intramitochondrial distribution of the APEX fusion proteins in the different strains used in this study. (G) The IMS-APEX and matrix-APEX reporters were fused to the eleventh beta sheet of GFP (GFP₁₁) and coexpressed with IMS- and matrix-targeted fragments of the first 10 beta sheets of GFP (GFP₁₋₁₀). Signals indicate colocalization with these split-GFP reporters (Cabantous and Waldo, 2006). Source data are available online for this figure.



◀ **Figure EV3. A nuclear model for mitochondrial dysfunction phenocopies the situation in $\Delta atp6$ cells.**

(A) Wild-type (WT) and $\Delta atp23$ cells were grown to log phase in galactose-containing medium before tenfold serial dilutions were dropped onto medium containing glucose or glycerol as carbon sources. (B) The growth of $\Delta atp23$ cells expressing the indicated APEX reporters was measured continuously. (C) Mitochondria were isolated from wild-type and $\Delta atp23$ cells and incubated with or without proteinase K (PK) to remove non-imported proteins. The presence of the surface protein Tom70 and of the FLAG-tagged APEX fusion proteins was verified by Western blotting. (D) Wild-type and $\Delta atp23$ cells were grown in galactose medium. Mitochondria were stained with the membrane potential-dependent dye mitotracker CMXRos. Intensity values were calculated to indicate the degree of mitochondrial energization in the two strains. (E) The fluorescence intensities of the mitotracker-stained mitochondria were quantified as a measure of the mitochondrial membrane potential in the strains. Shown are mean values and standard deviations from four biological replicates. (F) Comparison of the TargetP values of IMS-located MRPs in comparison to other matrix proteins that are enriched in the IMS of $\Delta atp23$ mitochondria. Shown are proteins that are significantly enriched $\log FC > 1$, P value < 0.05 . (G-I) Volcano plots showing the relative distribution of proteins purified with streptavidin beads in mitochondria from wild-type and $\Delta atp23$ cells. The $\log_2$ fold change ($\log FC$) values were calculated from $n = 4$ samples. Proteins of the small subunit of the mitoribosome are colored in red and of the large subunit in orange. Significantly enriched proteins are indicated by the dashed lines ($\log FC > 1$, P value < 0.05). Please note the strong accumulation of MRPs (shown in color) in the IMS of $\Delta atp23$ cells, that is reminiscent to the situation in $\Delta atp6$ cells (Fig. 2F). Source data are available online for this figure.

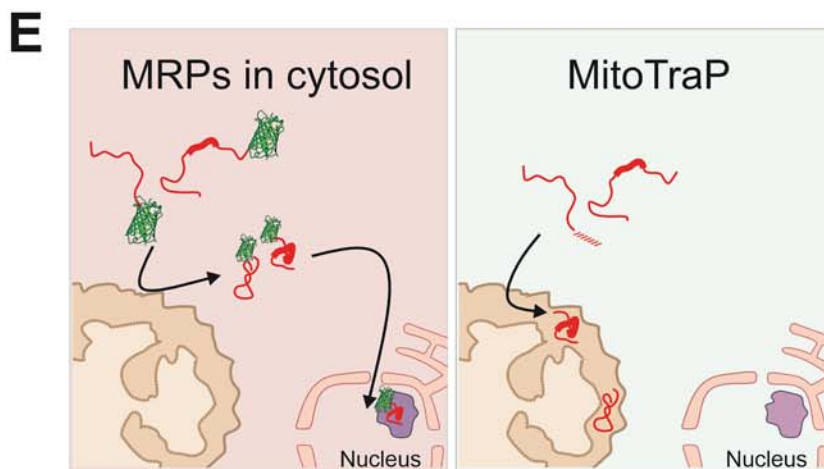
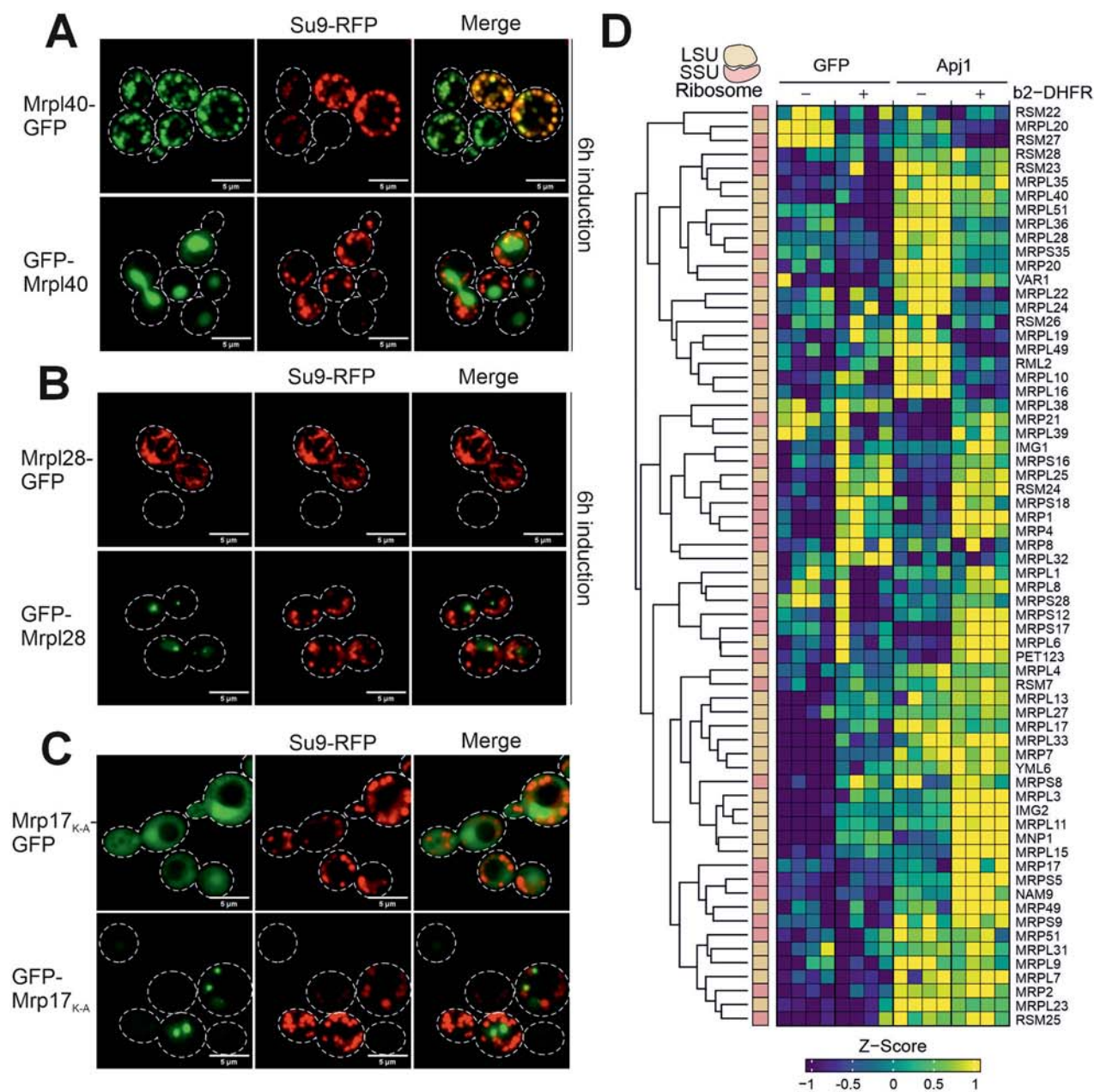


Figure EV5. The non-importable GFP-Mrp17 variant associates with components of the 90S pre-ribosome assembly complexes.

(A–C) GFP-Mrp140, GFP-Mrp128 and GFP-Mrp17_{K-A} were expressed and visualized by fluorescence microscopy 6 h after induction in galactose-containing medium. Mitochondria were stained by expression of Su9-RFP. (D) Heat map showing the intensities of MRPs there were co-isolated with GFP or Apj1-GFP in the presence or absence of the clogger b₂-DHFR. Proteins were filtered for small and large subunits of the mitochondrial ribosomes. Imputed LFQ intensities were z-score normalized across rows and subject to non-supervised hierarchical clustering. $n = 4$. (E) Mitochondrial dysfunction leads to the trapping of MRPs in the IMS. The accumulation of MRPs in the IMS might prevent their targeting to the nucleus and, thus, their interference with the assembly of ribosomes in the nucleolus.