

Supplementary Figures

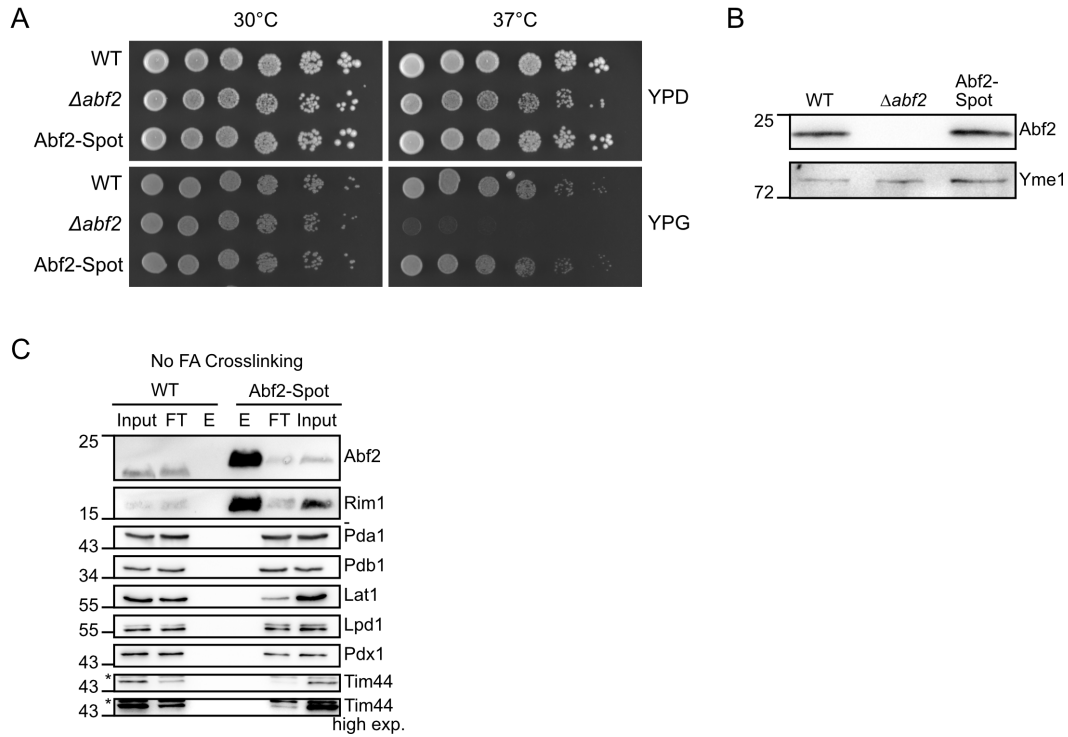


Figure S1: Abf2-Spot is functional and co-purifies PDH subunits only after crosslinking. **(A)** Growth analysis of wild-type (WT), $\Delta abf2$, and Abf2-Spot cells on YPD and YPG at 30 °C and 37 °C. **(B)** Immunoblot of whole-cell extracts from WT, $\Delta abf2$, and Abf2-Spot cells probed for Abf2 (Yme1, control). Molecular masses (kDa) are indicated at left. **(C)** Immunoblot of Abf2-Spot affinity purifications performed *without* formaldehyde crosslinking, comparing WT and Abf2-Spot mitochondria. Input, flow-through (FT), and eluate (E) fractions were probed with the indicated antibodies (Tim44 – higher-exposure below – control; asterisk, non-specific band). Molecular masses (kDa) are indicated at left.

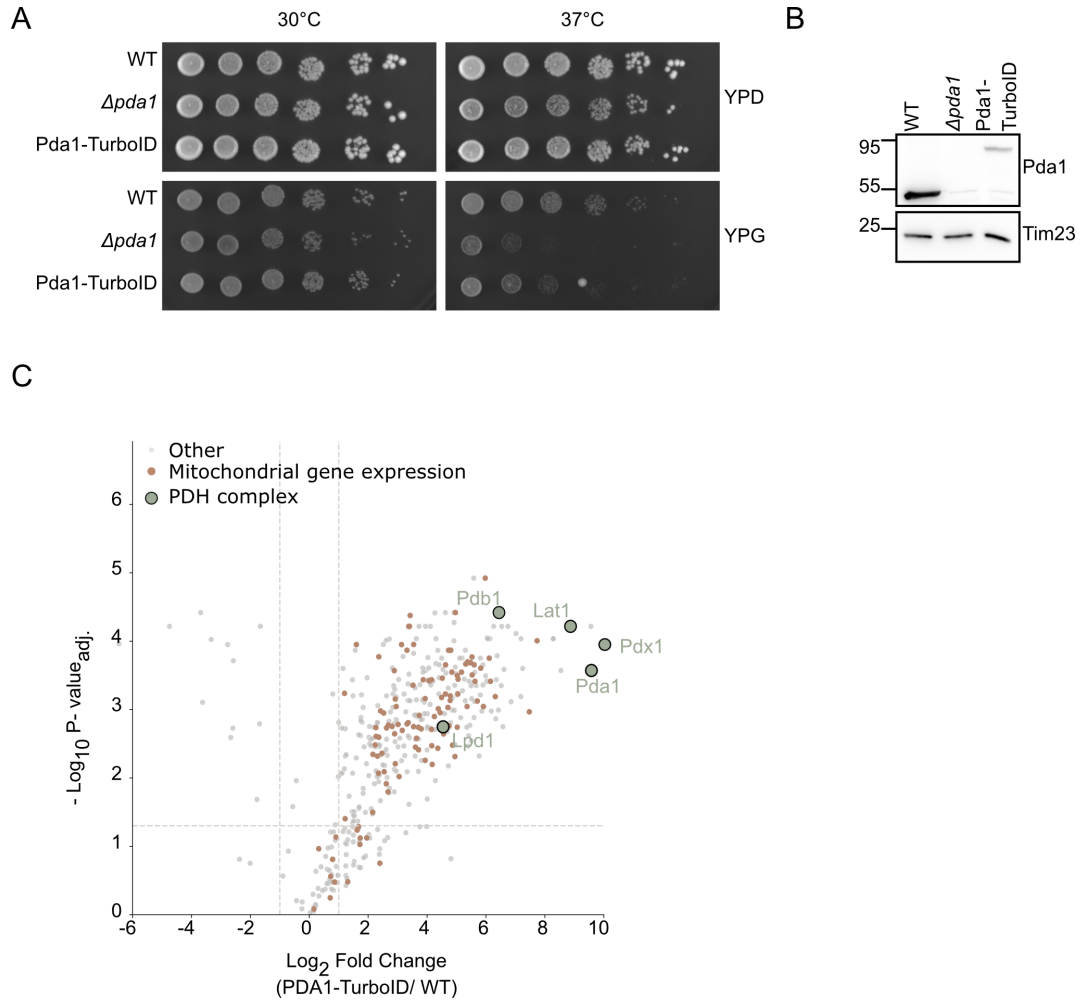


Figure S2: **Validation of the Pda1-TurboID fusion strain.** **(A)** Growth analysis of wild-type (WT), $\Delta pda1$, and Pda1-TurboID cells on YPD and YPG at 30 °C and 37 °C. **(B)** Immunoblot of whole-cell extracts from WT, $\Delta pda1$, and Pda1-TurboID cells probed for Pda1 (Tim23, control). Molecular masses (kDa) are indicated at left. **(C)** Volcano plot of streptavidin-purified proteins, Pda1-TurboID versus untagged wild-type control (Welch's *t*-test, Benjamini-Hochberg correction). PDH complex subunits (green) and mitochondrial gene-expression factors (orange) are highlighted; all other proteins are shown in grey. Dashed lines indicate the significance and fold-change thresholds ($q < 0.05$, $\log_2$ fold change > 1).

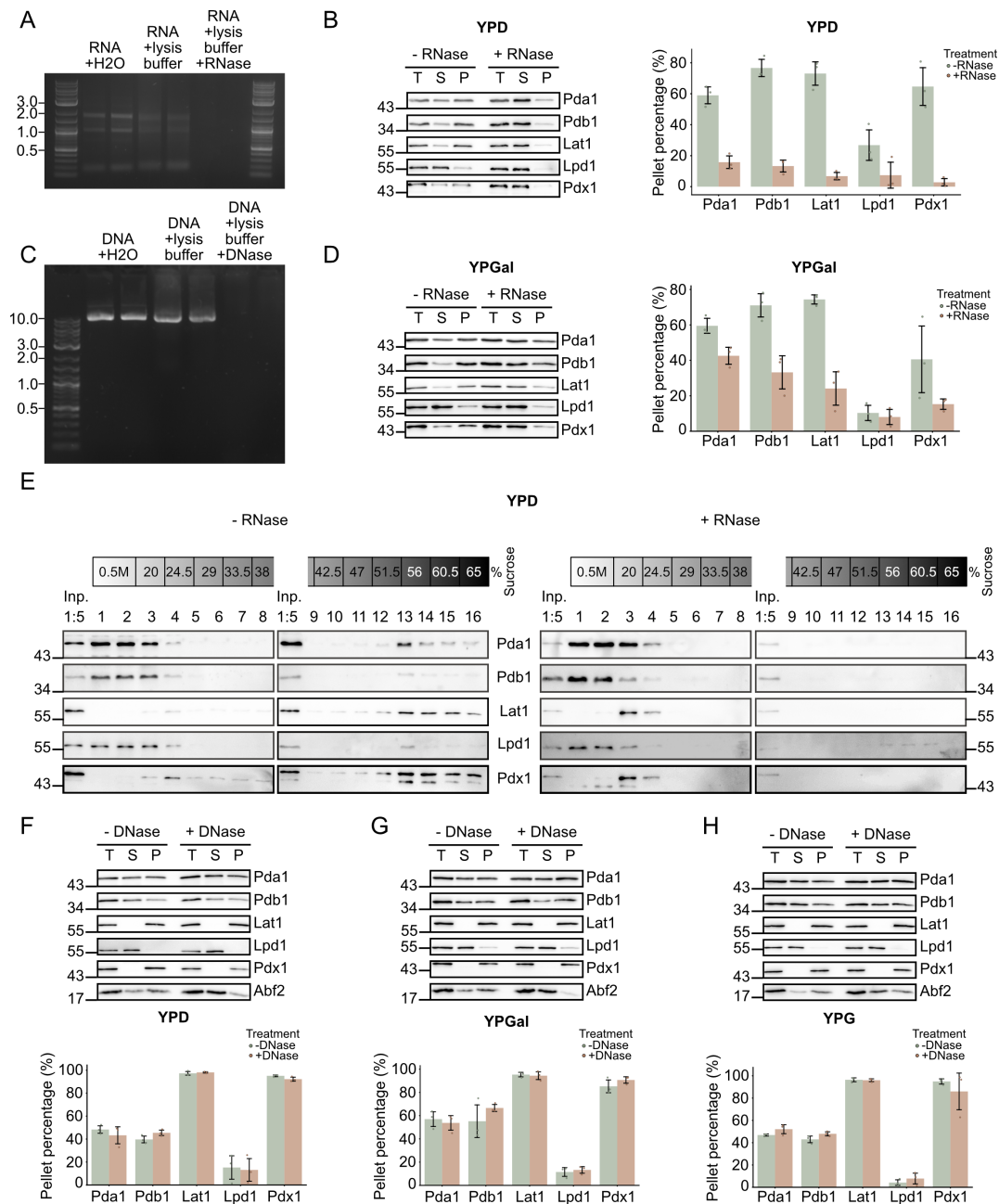


Figure S3: The pellet association of PDH is RNA-dependent and DNA-independent across carbon sources. (A) Agarose gel confirming RNase activity: purified mitochondrial RNA incubated in water, lysis buffer, or lysis buffer with RNase. DNA ladder sizes (kb) are indicated on the left. (B) Centrifugation-based fractionation of digitonin-solubilized mitochondria from YPD-grown cells into total (T), supernatant (S), and pellet (P) fractions, with or without RNase, analyzed by immunoblotting (left), with quantification of the pellet fraction (right). (C) Agarose gel confirming DNase activity: purified plasmid DNA incubated in water, lysis buffer, or lysis buffer with DNase. DNA ladder sizes (kb) are indicated on the left. (D) As in (B), for YPGal-grown cells. (E) Sucrose density gradient centrifugation (20–65%) of digitonin-solubilized mitochondria from YPD-grown cells, with or without RNase. Input and fractions 1–16 are shown. (F–H) Centrifugation-based fractionation as in (B), performed with or without DNase, for YPD (F), YPGal (G), and YPG (H) grown cells; immunoblots (top) and quantification of the pellet fraction (bottom). Abf2 is shown as a control. All quantifications: $n = 3$ biological replicates, mean $\pm$ SD.

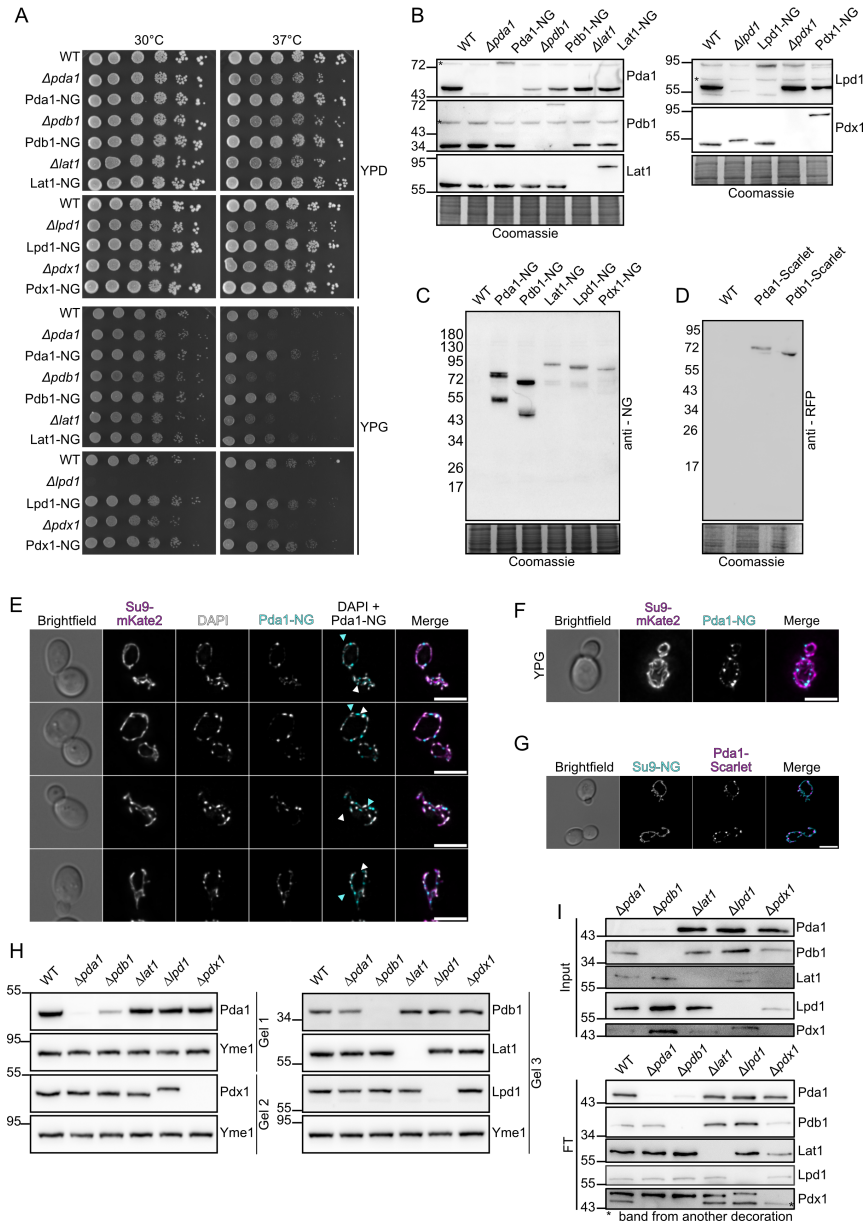


Figure S4: Validation of fluorescently tagged PDH subunits and supporting analyses of Pda1 foci. (A) Growth analysis of the indicated deletion strains and their mNeonGreen-tagged counterparts on YPD and YPG at 30 °C and 37 °C. (B) Immunoblots of the indicated PDH subunits in wild-type, deletion, and NG-tagged strains; Coomassie staining is shown as a loading control. Asterisks mark non-specific bands. (C) Anti-mNeonGreen (NG) immunoblot of cells expressing the indicated PDH subunit-NG fusions; Coomassie staining is shown as a loading control. All fusions are detected at their predicted full-length mass; a faster-migrating species (offset by ~23 kDa) is present for Pda1, Pdb1, Lat1, and Lpd1. (D) Anti-RFP immunoblot of cells expressing Pda1-mScarlet or Pdb1-mScarlet; Coomassie staining is shown as a loading control. Each fusion is detected as a single full-length species. (E) Additional Pda1-NG cells co-stained with DAPI, corresponding to Fig. 4F. Cyan arrowheads, Pda1-NG foci lacking a coincident DAPI signal; white arrowheads, DAPI-stained nucleoids with an adjacent Pda1-NG focus. Scale bars, 5 µm. (F) Fluorescence microscopy of Pda1-NG and Su9-mKate2 in YPG-grown cells. Scale bar, 5 µm. (G) Fluorescence microscopy of cells co-expressing Pda1-mScarlet and the matrix marker Su9-NG. Scale bar, 5 µm. (H) Immunoblots of the indicated PDH subunits in wild-type and PDH deletion strains (Yme1, control). (I) Immunoblots of input and flow-through (FT) fractions from the Lat1 immunoprecipitations shown in Fig. 4D, probed for the indicated PDH subunits. Asterisks mark bands from another decoration.

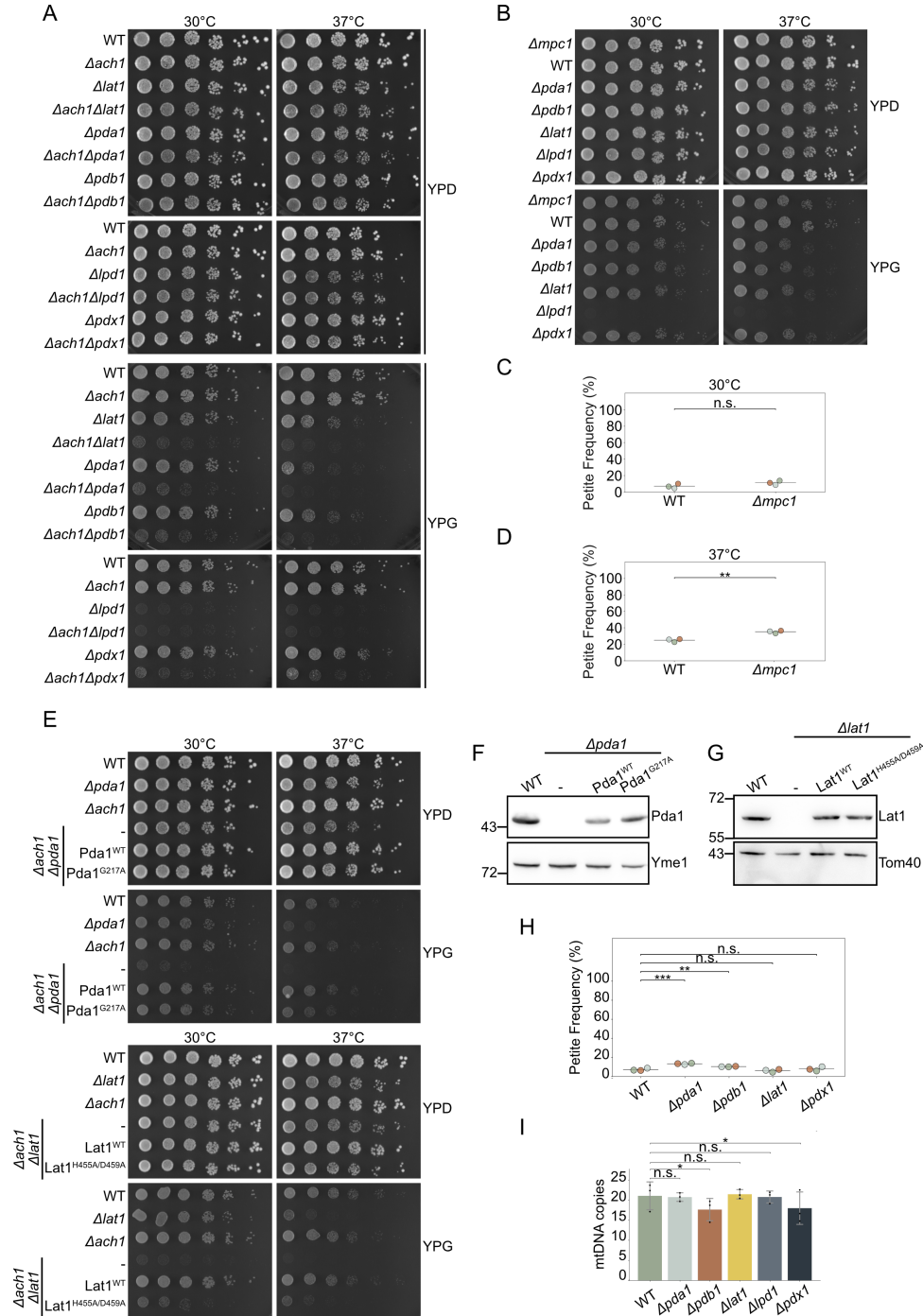


Figure S5: **Genetic interactions with *ACH1*, expression of catalytically inactive variants, and mtDNA phenotypes at 30 °C.** (A) Growth analysis of the indicated single and $\Delta ach1$ double mutants on YPD and YPG at 30 °C and 37 °C. (B) Growth analysis of $\Delta mpc1$ alongside wild-type and PDH deletion strains on YPD and YPG at 30 °C and 37 °C. (C, D) Petite frequency of wild-type and $\Delta mpc1$ cells at 30 °C (C) and 37 °C (D). (E) Growth analysis of $\Delta ach1 \Delta pda1$ and $\Delta ach1 \Delta lat1$ cells expressing no variant (–), the wild-type protein, or the catalytically inactive variant (Pda1^{G217A}, Lat1^{H455A/D459A}) on YPD and YPG at 30 °C and 37 °C. (F) Immunoblot of Pda1 in $\Delta pda1$ cells expressing no variant (–), Pda1^{WT}, or Pda1^{G217A} (Yme1, control). Molecular masses (kDa) are indicated at left. (G) Immunoblot of Lat1 in $\Delta lat1$ cells expressing no variant (–), Lat1^{WT}, or Lat1^{H455A/D459A} (Tom40, control). Molecular masses (kDa) are indicated at left. (H) Petite frequency of wild-type and the indicated PDH deletion strains at 30 °C. (I) Mitochondrial DNA copy number of wild-type and the indicated PDH deletion strains at 30 °C, determined by qPCR (*COX1/ACT1*). All quantifications: $n = 3$ biological replicates, mean $\pm$ SD.

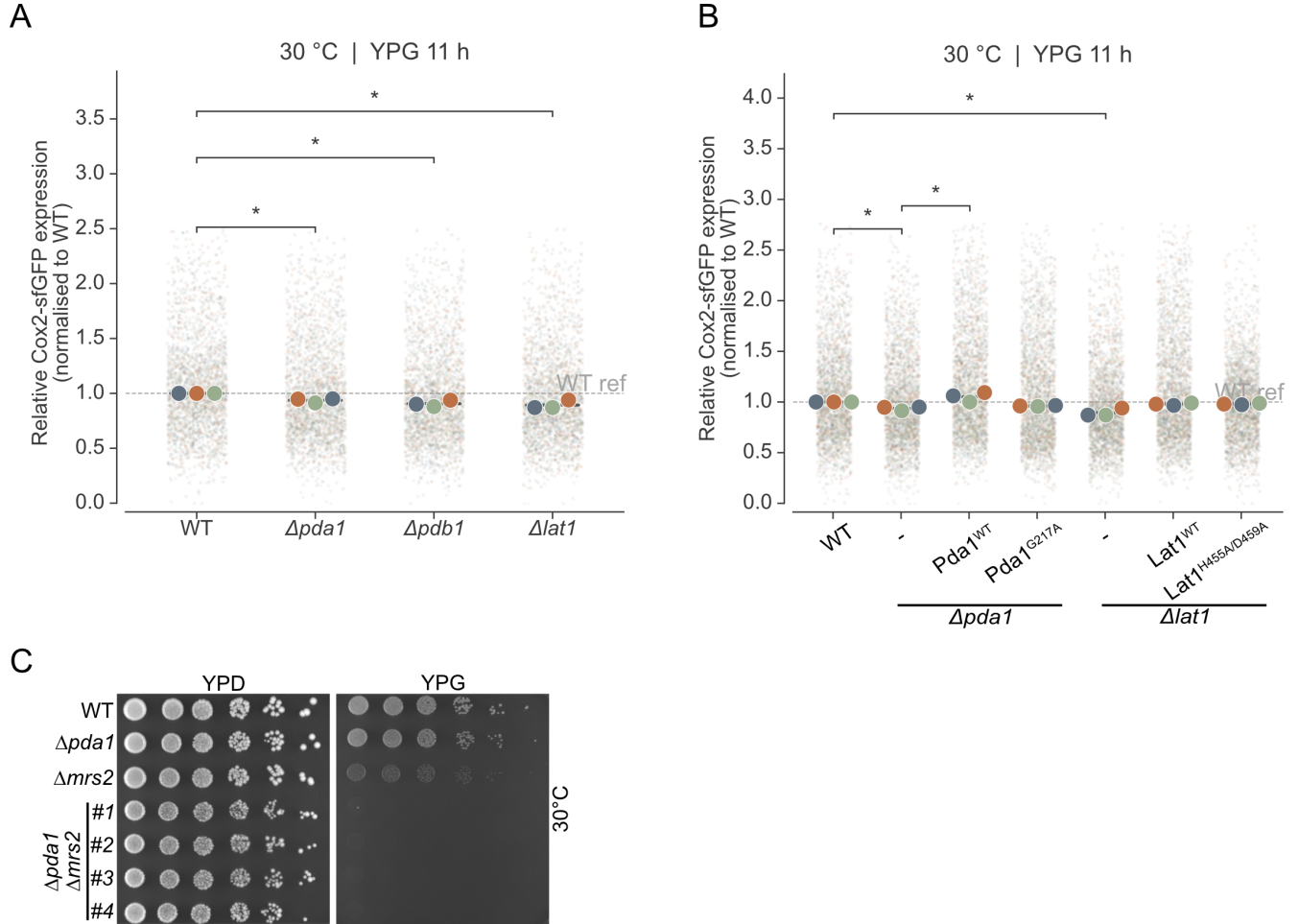


Figure S6: **Mitochondrial translation reporter output at 30 °C and genetic interaction of *PDA1* with *MRS2*.** (A) Cox2-sfGFP reporter expression in wild-type, $\Delta pda1$, $\Delta pdb1$, and $\Delta lat1$ cells grown in YPG for 11 h at 30 °C, normalized to wild-type (WT ref, dashed line). $n = 3$ biological replicates. (B) Cox2-sfGFP reporter expression in $\Delta pda1$ and $\Delta lat1$ cells expressing no variant (–), the wild-type protein, or the catalytically inactive variant ($Pda1^{G217A}$, $Lat1^{H455A/D459A}$), grown as in (B) and normalized to wild-type. Plotted as in (B). $n = 3$ biological replicates. (C) Growth analysis of wild-type, $\Delta pda1$, $\Delta mrs2$, and four independent $\Delta pda1 \Delta mrs2$ double-mutant segregants (#1–4), each from a separate tetrad, on YPD and YPG at 30 °C. Cox2-sfGFP reporter expression in $\Delta pda1$ and $\Delta lat1$ cells expressing no variant (–), the wild-type protein, or the catalytically inactive variant ($Pda1^{G217A}$, $Lat1^{H455A/D459A}$), grown as in (B) and normalized to wild-type. Plotted as in (B). $n = 3$ biological replicates.