Integrative Proteomics and Biochemical Analyses Define Ptc6p as the *Saccharomyces cerevisiae* Pyruvate Dehydrogenase Phosphatase

Xiao Guo, Natalie M. Niemi, Joshua J. Coon, and David J. Pagliarini

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids and primers used in this study—Standard restriction cloning or polymerase incomplete primer extension (PIPE) cloning was used to generate the plasmids. A FLAG tag was cloned into the yeast expression vector p416 GPD through PIPE cloning (1,2). The *ptc5*, *ptc6*, *ptc7*, *coq8*, and *coq9* gene were cloned into the C-terminal FLAG tagged p416 GPD vector using SpeI and XhoI (for *coq8*), or XbaI and XhoI (for the rest of the genes) restriction sites. A bacterial expression vector pVP68K was used for protein purification, which encodes corresponding proteins fused to an 8xHis and maltose-binding protein (MBP) tags with a linker including a tobacco etch virus (TEV) protease recognition site (8xHis-MBP-[TEV]-Protein) (3). Full-length *ptc5*, full-length *ptc6*, *ptc7* with N-terminal deletion of 38 residues (Nd38), and *hfd1* with C-terminal deletion of 25 residues (Cd-25) were cloned into pVP68K using the PIPE cloning method (2). The *Pda1*-containing plasmids used to verify the phospho-antibody are in a p416-based vector containing a C-terminal 6xHis and 3x HA tag (a gift from Jared Rutter). Pda1p with its endogenous promoter (~700 bp upstream of the gene) was cloned into this vector using SacI and SpeI restriction sites. S313A and S315A mutants were generated using site-directed mutagenesis (SDM) with PfuUltra high-fidelity DNA polymerase AD (Agilent) with the following primers: S313A fwd: TACGGTGGCCATGCTATGTCTGATC, S313A rev: GATCAGACATAGCATGGCCACCGTA; S315A fwd: GTACGGTGGCCATTCTATGGCTGATCCCGGTACTACCTAC, S315A rev: GTAGGTAGTACCGGGATCAGCCATAGAATGGCCACCGTAC. The primers for endogenous HA tagging are: fwd: TACTTGGGACTTCAAAAAGCAAGGTTTTGCCTCTAGGGATCGGATCCCCGGGTTAATTAA, rev: CGATCACAGCACTATTATTTTATTTTTCCTTACGATTTAAGAATTCGAGCTCGTTTAAAC.

Western blotting—A standard western blotting protocol was done for SDS-PAGE gels. All primary antibodies used are listed here: phospho-S313 of Pda1p (Abcam, ab92696 with 1:500 dilution), VDAC (Abcam, ab110326 with 1: 1,000 dilution), HA (Thermo, 26183 with 1:10,000 dilution), and FLAG (M2, Sigma-Aldrich, F1804 with 1:5,000 dilution). Secondary antibodies were used at a 1:10,000 dilution. The LI-COR Odyssey CLx imaging system was used to visualize protein bands, and Image Studio was used to analyze the image and calculate band intensity. Bovine serum albumin was used instead of non-fat dried milk as a blocking reagent when working with phospho-specific antibody.

Isolation of crude mitochondria from yeast—The protocol for isolation of crude mitochondria from yeast is adapted as previously described (4). Yeast were streaked out from a glycerol stock onto a YEPD plate (2% bacto peptone, 1% yeast extract, 2% dextrose, all in w/v) supplemented with G418 and grown for 2 days at 30°C. Individual colonies were picked into 3mL YEPD starter cultures and grown overnight at 30°C with 230 rpm shaking. 1×10^8 of cells were inoculated into 1L YEPD medium and allowed to grow at 30°C overnight. OD_{600} was monitored and yeast were harvested around 4 h after diauxic shift. Cells were collected by centrifugation at 2,000 × g for 5 min. The supernatant was discarded and wet weight was measured. The cell pellet was resuspended in 30 mL of tris(hydroxymethyl)aminomethane (Tris)-dithiothreitol (DTT) buffer (0.1 M Tris-SO4, 10 mM DTT, pH 9.4) and incubated for 15 min at 30°C with shaking at 230 rpm. The cell suspension was centrifuged for 5 min at 1,500 x g. The cell pellet was resuspended in 30 mL SP buffer (1.2 M sorbitol, 20 mM KH_2PO_4-K_2HPO_4, pH 7.4) and centrifuged again for 5 min at 1,500 × g to wash out leftover Tris-DTT buffer. The cell pellet was resuspended in 30 mL SP buffer and transferred into a 250 mL Erlenmeyer flask. Zymolyase 20 T (from *Arthrobacter luteus*, MP Biomedicals) was added according to the wet weight of cell pellet (7.5 mg zymolyase per gram of wet cells) to digest the yeast cell walls. The suspension was shaken gently (200 rpm) at 30°C for 40 min. The spheroplasts were pelleted by centrifugation at 4,500 × g for 5 min. All subsequent steps were carried at 4°C or on ice. The spheroplasts were washed once with ice-cold SEH buffer (0.6 M sorbitol, 20 mM 4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH, 2 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM phenylmethane sulfonyl fluoride (PMSF), with protease inhibitor and phosphatase inhibitor). The pellet was resuspended in 30 mL SEH buffer and poured into a 40 mL glass/glass Dounce homogenizer. 15 strokes were used to homogenize the suspension with a tight-fitting pestle. The homogenate was centrifuged at 1,500 × g (low speed) for 5 min to pellet nuclei, cell debris, and unbroken cells. The supernatant was centrifuged at 12,000 × g for 10 min to pellet mitochondria. The low-speed pellet was subjected to another round of homogenization followed by low-speed and high-speed centrifugation. The two high-speed pellets were combined, and resuspended in 30 mL SEH buffer using Dounce homogenizer with a loose-fitting pestle. The suspension was subjected to another round of low-speed and high-speed centrifugation. The pellet was resuspended in SEH buffer, aliquoted into 1.5 mL Eppendorf tubes, flash frozen in liquid nitrogen, and stored in -80°C for future use.

MS sample preparation for proteomics—Crude mitochondria were thawed on ice and resuspended in lysis buffer (8 M urea, 40 mM Tris, 30 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, with cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, 11836170001 ROCHE), and PhosSTOP™ (Sigma-Aldrich, PHOSS-RO ROCHE), pH 8.0) (5). Three rounds of sonication (10 s on, 30 s off) were done to lyse the sample. Bicinchoninic acid (BCA) assay was performed to determine protein concentration. 500 µg of protein from each sample was taken, and DTT was added to a final concentration of 2 mM for reducing reaction with incubation at 37°C for 30 min. Iodoacetimide (IAA) was added to a final concentration of 7 mM for alkylation reaction with incubation at room temperature in the dark for 30 min. DTT was added to achieve a final total concentration of 7 mM to quench the extra IAA. Lys-C was added to the sample (1:100 mass ratio to total protein) followed by incubation at 37°C for 4 h. 50 mM Tris (pH 8.0) and 1 mM $CaCl_2$ were added to the samples to dilute the concentration of urea to 1.5 M. Trypsin was added (1:100 mass ratio to total protein) followed by incubation at 37°C overnight. 10% trifluoroacetic acid (TFA) was added to bring the pH below 2 in order to quench the digestion reaction. The peptides were resuspended in 1 mL 0.1% TFA, and desalted using Sep-Pak Vac 1cc tC18 cartridges (Waters). The desalting protocol contains four steps: column equilibration, sample loading, wash, and elution. The columns were equilibrated with 3 mL acetonitrile (ACN), 1 mL 70% ACN, 1mL 40% ACN, 1 mL 20% ACN, and 3 mL 0.1% TFA. The wash was done with 3 mL 0.1% TFA after sample loading. The elution was done by adding 1 mL 40% ACN, and then 0.75 mL 70% ACN. After drying down in a Speed Vac, the samples were resuspended in 100 µL of 200 mM triethyl ammonium bicarbonate (TEAB, pH 8.0) followed by 6-plex Tandem Mass Tag (TMT, Thermo) labeling. The TMT reagent was resuspended in 50 µL ACN. Each tag was mixed with the peptide sample, and incubated at room temperature for 2 h with shaking. 8 µL of 5% hydroxylamine was added to each sample followed by incubation at room temperature for 15 min with shaking. The six samples were mixed together and dried down followed by strong cation exchange (SCX) chromatography for fractionation.

Four buffers were used in SCX chromatography: buffer A: 5 mM KH_2PO_4 in 30% ACN, pH 2.65; buffer B: 5 mM KH_2PO_4, 350 mM KCl in 30% ACN, pH 2.65; buffer C: 50 mM KH_2PO_4, 500 mM KCl in H_2O, pH 6.5; and buffer D: nanopure H_2O. Dried samples were resuspended in buffer A and loaded to a polysulfoethylaspartamide column. 8 fractions were collected during a 60 min gradient. Each fraction was lyophilized and subjected for desalting followed by phosphopeptide enrichment. Phosphopeptide enrichment was done with immobilized metal affinity chromatography (IMAC) using magnetic agarose beads (Qiagen) (6). The magnetic beads were washed three times with water followed by incubation in 40 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0) for 30 min with shaking. The beads were then washed another three times with water. 100 mM $FeCl_3$ was added to the beads and incubated for 30 min with shaking. The beads were further washed with 80% ACN with 0.1% TFA for four times before loading samples. The dried samples were resuspended in 1 mL 80% ACN with 0.1% TFA, and loaded to the beads followed by 30 min incubation with shaking. The flow-through fraction was saved for total protein analysis, and the beads were washed three times with 80% ACN with 0.1% TFA. 100 µL of 50% ACN with 0.7% NH_4OH was added to the beads and vortexed for 1 min to elute phosphopeptides. 4% formic acid was added to the elution before drying down.

LC-MS/MS and data analysis—All samples were analyzed by reverse phase liquid chromatography on an EASY-nLC system (Thermo) coupled to a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometry (Thermo) as previously described (7,8). The reverse phase column with 75-360 µm inner-outer diameter was packed with 1.7 µm C18 particles. A 90 min gradient was run at 60°C with two mobile phases (A: 0.1% formic acid, and 5% dimethyl sulfoxide (DMSO) in H_2O; B: 0.1% formic acid, and 5% DMSO in ACN). MS^1 scans were performed at 35 K resolution from 300 to 1500 m/z, and the top 15 most abundant precursors were isolated in a 2.0 Th window for Higher-energy collisional dissociation (HCD) fragmentation with normalized collision energy of 28 or 30. The MS^2 scans were performed at resolution of 17.5 K. The AGC target was set at 1e6 for both MS^1 and MS^2. The maximum injection time was set at 120 ms for MS^1 and 60 ms for MS^2. 30 s of dynamic exclusion time was applied, and precursors with +1 or unassigned charge states were excluded from MS^2.

The Coon OMSSA Proteomics Software Suite (COMPASS) was used to analyze the MS raw files (9). *DTA generator* was applied to convert raw data files into .dta text files for searching. The Open Mass Spectrometry Search Algorithm (OMSSA) was applied to search files against a target-decoy database of both canonical and isoforms of *Saccharomyces cerevisiae* proteome downloaded from UniProt. Carbamidomethylation of cysteines, and 6-plex TMT labeling on N-terminus and lysines were set as fixed modifications. Oxidation of methionines and 6-plex TMT on tyrosines were set as variable modifications. Phosphorylation with neutral loss on serine or threonine, and intact phosphorylation on tyrosine was added as variable modifications for phosphopeptide searching. Other searching parameters were set up as: a precursor mass tolerance of 150 ppm, a product ion mass tolerance of 0.01 Da, and up to 3 missed cleavages with trypsin digestion. *FDR Optimizer* was utilized to filter peptides to 1% false discovery rate (FDR) with maximum 25 ppm mass error. *TagQuant* was applied to calculate quantitative values of all peptide spectral matches (PSMs) using TMT reporter ion intensities with isotope purity corrections and normalization to total intensities in each channel. *Protein Hoarder* was used to group peptide to parsimonious protein groups at 1% FDR, and total reporter ion intensities of all unshared PSMs (excluding phospho-PSMs) were used as protein intensities. *Phosphinator* was used to assign the phosphorylation localization for phospho-PSMs with an ambiguity score threshold of 13, and total reporter ion intensities of all localized phospho-PSMs represented the intensity of the phosphoisoform. Proteins and phosphoisoforms were assigned as either mitochondrial or non-mitochondrial ones based on the yeast MitoMiner database (http://mitominer.mrc-mbu.cam.ac.uk/release-3.0/begin.do) (10). The intensities of phosphoisoforms from different channels were further normalized to corresponding protein intensities. Hierarchical clustering was done using Perseus with Euclidean distance and average linkage (11).

Protein interaction study—Each C-terminally FLAG tagged protein construct (*ptc5*, *ptc6*, *ptc7*, *coq8*, and *coq9*) in p416 GPD plasmid with uracil (ura) selection under GPD promoter was transformed into wild-type BY4741 yeast. An empty vector was included as negative control. Yeast cells were grown in 100 mL uracil dropout (ura-) media with 1% dextrose (D, w/v) and harvested around 4 h after diauxic shift. The pellets were flash frozen in liquid nitrogen and stored in -80°C until ready to use. The following immunoprecipitation procedure was carried either on ice or at 4°C. After thawing the pellets on ice, lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 3% digitonin (w/v), with cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, 11836170001 ROCHE), and PhosSTOP™ (Sigma-Aldrich, PHOSS-RO ROCHE), pH 7.4) was added to resuspend the pellets. Glass beads were added, and the samples were beaten on a master beater for six rounds with each round 1 min and 30 s rest between. Lysate was transferred to a new tube, and clarified at 16,000 × g. Anti-FLAG M2 magnetic beads (Sigma) were washed three times with lysis buffer, and aliquoted into the supernatant of each sample. The samples were incubated with Anti-FLAG beads for 2 h on an end-to-end shaker. After removing the flow-through, the beads were washed three times with wash buffer 1 (50 mM Tris-HCl, 150 mM NaCl, 0.05% digitonin (w/v), with protease inhibitors, pH 7.4), and then twice with wash buffer 2 (50 mM Tris-HCl, 150 mM NaCl, with protease inhibitors, pH 7.4). Elution buffer (0.2 g/L FLAG peptide in wash buffer 2) was added, and beads were incubated at room temperature for 30 min with periodic agitation. The elution was then prepared for MS analysis in a similar manner as the proteomics sample preparation. MS lysis buffer (8 M urea, 40 mM, 30 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, with cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail

(Sigma-Aldrich, 11836170001 ROCHE), and PhosSTOP™ (Sigma-Aldrich, PHOSS-RO ROCHE), pH 8.0) was added to the elution to make the final urea concentration 6 M. DTT was added to a final concentration of 2 mM and samples were incubated at 37°C for 30 min. IAA was added to a final concentration of 7 mM, and samples were incubated at room temperature in the dark for 30 min. DTT was then added to achieve a final total concentration of 7 mM to quench the IAA. The samples were diluted with 50 mM Tris (pH 8.0) and 1 mM $CaCl_2$ to achieve a final urea concentration of 1.5 M. Trypsin was added (1:100 mass ratio to total protein) followed by incubation at 37°C overnight. 10% TFA was added to bring the pH below 2 to quench the digestion reaction. The samples were subjected to Sep-Pak for desalting. The dried samples were resuspended in 0.2% formic acid and ready for LC-MS/MS. The samples were run on a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo) with a 60 min gradient (12). MS^1 scans were performed at 70 K resolution from 300 to 1500 m/z, and top 20 most abundant precursors were isolated in a 2.0 Th window for HCD fragmentation with normalized collision energy of 25. The MS^2 scans were performed at resolution of 17.5 K. The AGC target was set at 3e6 MS^1 and 1e5 for MS^2. The maximum injection time was set at 20 ms for MS^1 and 60 ms for MS^2. 20 s of dynamic exclusion time was applied, and precursors with +1 or unassigned charge states were excluded from MS^2. The raw files were analyzed by COMPASS (9). WD score was calculated as previously described with PSM used for PPI intensity (12,13). A 90% cutoff was used to filter out interactions, and manual check was done to pick up PPIs with low but specific PSMs.

Protein Purification—Recombinant proteins were purified as previously described with some minor modifications (14,15). pVP68K plasmids containing the corresponding protein gene were transformed in to BL21-CodonPlus(DE3)-RIPL strain (Agilent Technologies) with chloramphenicol (cam) and kanamycin (kan) selection. Single colonies were picked into Lysogeny broth (LB) starter culture supplemented with cam/kan and grown overnight at 37°C. The whole starter culture was transferred into 500 mL TB+G AIM auto-induction media (12 g/L tryptone, 24 g/L yeast extract, 12.8 g/L glycerol, 89 mM KH_2PO_4/K_2HPO_4, 0.375% aspartate, 0.15 g/L glucose, 5 g/L α-lactose monohydrate, 2 mM $MgSO_4$, and cam/kan), and grown for 2 h at 37°C then switched to 25°C for 18 h. The cultures were harvested by centrifugation at 4,000 × g for 20 min, transferred to 50 mL Falcon tubes, flash frozen, and stored at -80°C until ready for protein purification. All subsequent steps were carried at 4°C or on ice. Pellets were resuspended in 30 mL lysis buffer (50mM HEPES, 300 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol (BME), 0.25 mM PMSF, 1g/L lysozyme, pH 7.5), and sonicated 4 times for 20 s with 60 s rest between sonication rounds (75% amplitude). The cell lysate was clarified by centrifugation at 15,000 × g for 30 min. The supernatant was incubated with Talon resin (Clontech Laboratories) for 1 h on a nutator. The resin was pelleted by centrifugation at 700 × g for 5min, and the supernatant was discarded. The resin was washed 4 times with wash buffer (50mM HEPES, 300 mM NaCl, 10% glycerol, 5 mM BME, 0.25 mM PMSF, 10 mM imidazole, pH 7.2). His-tagged proteins were eluted from the resin by mixing with elution buffer (50mM HEPES, 300 mM NaCl, 10% glycerol, 5 mM BME, 0.25 mM PMSF, 100 mM imidazole, pH 7.2). The eluates were transferred to primed MW-cutoff spin column (Amicon) to be concentrated and exchanged to equilibration/storage (Eq/S) buffer (50mM HEPES, 300 mM NaCl, 10% glycerol, 5 mM BME, 10 mM imidazole, pH 7.2). The concentration of protein of the eluate was quantified by A280 using Nanodrop, and TEV protease was added (1:50, TEV/fusion protein, mass:mass) to cut off both His and MBP tags. A second subtractive IMAC was done by adding the TEV reaction mixture to the Talon resin followed by incubation for 1h. The supernatant was transferred to MW-cutoff spin column and concentrated to ~ 1 mL. The proteins were aliquoted, flash frozen and stored in -80°C. The concentration of protein was determined by quantification of the intensity of corresponding band after Coomassie staining with BSA standards.

Pyruvate dehydrogenase complex (PDC) activity assay—Purified mitochondria were lysed in lysis buffer (150 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 3 mM $MgCl_2$, 0.03 mM $CaCl_2$, 0.25% Triton X-100 (v/v), with cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, 11836170001 ROCHE), and PhosSTOP™ (Sigma-Aldrich, PHOSS-RO ROCHE), pH 7.4) by pipetting up and down several times. The insoluble material was pelleted by centrifugation at 16,000 × g for 10 min. The supernatant was taken and subjected to BCA to determine protein concentration. The supernatant was

further diluted to a protein concentration of 0.5 g/L for activity assay. The pyruvate dehydrogenase complex assay was done on a 96-well plate (UV star) in a Cytation 3 plate reader at 30°C. Each well contains 50 µL reaction buffer (150 mM MOPS, 3 mM $MgCl_2$, 0.03 mM $CaCl_2$, pH 8.0), 20 µL of 3 mM thiamine pyrophosphate (TPP), 20 µL of 25 mM β-Nicotinamide adenine dinucleotide sodium salt (NAD+), 20 µL of 38mM cysteine (pH 7.4), 20 µL of 50 mM sodium pyruvate, and 50 µL of mitochondrial lysate (16). 20 µL of 1.5 mM Coenzyme A sodium salt was added to initiate the reaction. A340nm was monitored to calculate nicotinamide adenine dinucleotide (NADH) production rate.

In vitro phosphatase assays—In vitro phosphatase assay on para-nitrophenyl phosphate (pNPP, New England Biolabs) was done in a 96-well plate using a Cytation 3 plate reader (BioTek). 30 µL of 50 ng/µL protein was added to 150 µL reaction buffer (66.7 mM Tris, 13.3 mM $MnCl_2$, pH 8.0). The reaction was initiated by addition of 20 µL of 500 mM pNPP, and A405nm was recorded over time. The initial slope was calculated to represent the reaction rate.

For *in vitro* phosphatase assay on Pda1p, purified mitochondria from Δ*ptc6* were lysed in 1 mL lysis buffer (50 mM Tris, 150 mM NaCl, 0.25% Triton X-100, with cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, 11836170001 ROCHE), pH 7.4) by pipetting up and down. The supernatant was aliquoted into 350 µL per tube after spin at 16,000 × g for 10 min to pellet the insoluble materials. 200 µL of 100 mM $MnCl_2$ and 0.2 µL of 100 mM $CaCl_2$ were added into each aliquote before addition of 60 µL of 50 ng/µL recombinant protein. The mixture was incubated at room temperature. 20 µL of sample was taken out at different time points, mixed with 4 × sample buffer, and boiled at 95°C for 5min for western blotting. Another 10 µL of sample after 30 min treatment was used for the PDC activity assay (see above for method).

Oxygen consumption assays—Oxygen consumption assays for yeast were performed using a Seahorse analyzer as previously described (14). Yeast were freshly streaked onto 2% dextrose (D, w/v) containing plate and grown for 2 days at 30°C. Individual colonies were selected into synthetic complete (SC) 2%D liquid media (~3-4 mL) and incubated overnight (~14-16 h, 30°C, 230 rpm). Cells were normalized to 4×10^6 cells/mL in fresh SC 2% pyruvate (w/v) media, and 100 µL of these cells (final [c] cells = 4×10^5 cells/well) were transferred to a poly-D-lysine coated (25 µg/mL) 96 well Seahorse plate (Seahorse Biosciences). Plate was spun for 1 min at 1,000 × g to sediment yeast, which were then incubated at 30°C for 1 h in a non-CO2 incubator before being placed in the Seahorse XF-96 Extracellular Flux Analyzer. Oxygen consumption rate (OCR) was measured with a 1 min mix step and 5 min measure step for 10 rounds, with the final round of OCR reported.

SUPPLEMENTAL REFERENCES

1. Mumberg, D., Müller, R., and Funk, M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156, 119-122
2. Klock, H. E., and Lesley, S. A. (2009) The Polymerase Incomplete Primer Extension (PIPE) method applied to high-throughput cloning and site-directed mutagenesis. High Throughput Protein Expression and Purification: Methods and Protocols, 91-103
3. Blommel, P. G., Martin, P. A., Seder, K. D., Wrobel, R. L., and Fox, B. G. (2009) Flexi vector cloning. High Throughput Protein Expression and Purification: Methods and Protocols, 55-73
4. Boldogh, I. R., and Pon, L. A. (2007) Purification and subfractionation of mitochondria from the yeast Saccharomyces cerevisiae. Methods in cell biology 80, 45-64
5. Grimsrud, P. A., Carson, J. J., Hebert, A. S., Hubler, S. L., Niemi, N. M., Bailey, D. J., Jochem, A., Stapleton, D. S., Keller, M. P., Westphall, M. S., Yandell, B. S., Attie, A. D., Coon, J. J., and Pagliarini, D. J. (2012) A quantitative map of the liver mitochondrial phosphoproteome reveals posttranslational control of ketogenesis. Cell metabolism 16, 672-683
6. Phanstiel, D. H., Brumbaugh, J., Wenger, C. D., Tian, S., Probasco, M. D., Bailey, D. J., Swaney, D. L., Tervo, M. A., Bolin, J. M., Ruotti, V., Stewart, R., Thomson, J. A., and Coon, J. J. (2011) Proteomic and phosphoproteomic comparison of human ES and iPS cells. Nature methods 8, 821-827

7. Hebert, A. S., Richards, A. L., Bailey, D. J., Ulbrich, A., Coughlin, E. E., Westphall, M. S., and Coon, J. J. (2014) The one hour yeast proteome. Molecular & Cellular Proteomics 13, 339-347
8. Tagliabracci, V. S., Wiley, S. E., Guo, X., Kinch, L. N., Durrant, E., Wen, J., Xiao, J., Cui, J., Nguyen, K. B., Engel, J. L., Coon, J. J., Grishin, N., Pinna, L. A., Pagliarini, D. J., and Dixon, J. E. (2015) A single kinase generates the majority of the secreted phosphoproteome. Cell 161, 1619-1632
9. Wenger, C. D., Phanstiel, D. H., Lee, M., Bailey, D. J., and Coon, J. J. (2011) COMPASS: a suite of pre-and post-search proteomics software tools for OMSSA. Proteomics 11, 1064-1074
10. Smith, A. C., Blackshaw, J. A., and Robinson, A. J. (2011) MitoMiner: a data warehouse for mitochondrial proteomics data. Nucleic acids research, gkr1101
11. Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M., and Cox, J. (2016) The Perseus computational platform for comprehensive analysis of (prote) omics data. Nature methods 13, 731-740
12. Floyd, B. J., Wilkerson, E. M., Veling, M. T., Minogue, C. E., Xia, C., Beebe, E. T., Wrobel, R. L., Cho, H., Kremer, L. S., Alston, C. L., Gromek, K. A., Dolan, B. K., Ulbrich, A., Stefely, J. A., Bohl, S. L., Werner, K. M., Jochem, A., Westphall, M. S., Rensvold, J. W., Taylor, R. W., Prokisch, H., Kim, J.-J. P., Coon, J. J., and Pagliarini, D. J. (2016) Mitochondrial protein interaction mapping identifies regulators of respiratory chain function. Molecular cell 63, 621-632
13. Behrends, C., Sowa, M. E., Gygi, S. P., and Harper, J. W. (2010) Network organization of the human autophagy system. Nature 466, 68-76
14. Guo, X., Niemi, N. M., Hutchins, P. D., Condon, S. G., Jochem, A., Ulbrich, A., Higbee, A. J., Russell, J. D., Senes, A., Coon, J. J., and Pagliarini, D. J. (2017) Ptc7p Dephosphorylates Select Mitochondrial Proteins to Enhance Metabolic Function. Cell Reports 18, 307-313
15. Stefely, J. A., Reidenbach, A. G., Ulbrich, A., Oruganty, K., Floyd, B. J., Jochem, A., Saunders, J. M., Johnson, I. E., Minogue, C. E., Wrobel, R. L., Barber, G. E., Lee, D., Li, S., Kannan, N., Coon, J. J., Bingman, C. A., and Pagliarini, D. J. (2015) Mitochondrial ADCK3 employs an atypical protein kinase-like fold to enable coenzyme Q biosynthesis. Molecular cell 57, 83-94
16. Brown, J. P., and Perham, R. N. (1976) Selective inactivation of the transacylase components of the 2-oxo acid dehydrogenase multienzyme complexes of Escherichia coli. Biochemical Journal 155, 419-427

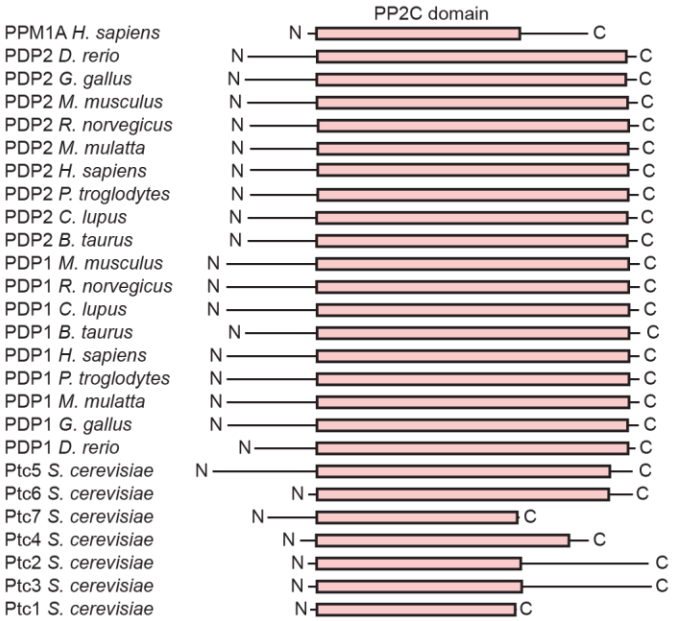

Fig. S1. PP2C domains alignment. A schematic representation of the PP2C domains in vertebrate PDP1 and PDP2, human PPM1A, and seven *Saccharomyces cerevisiae* PP2C phosphatases.

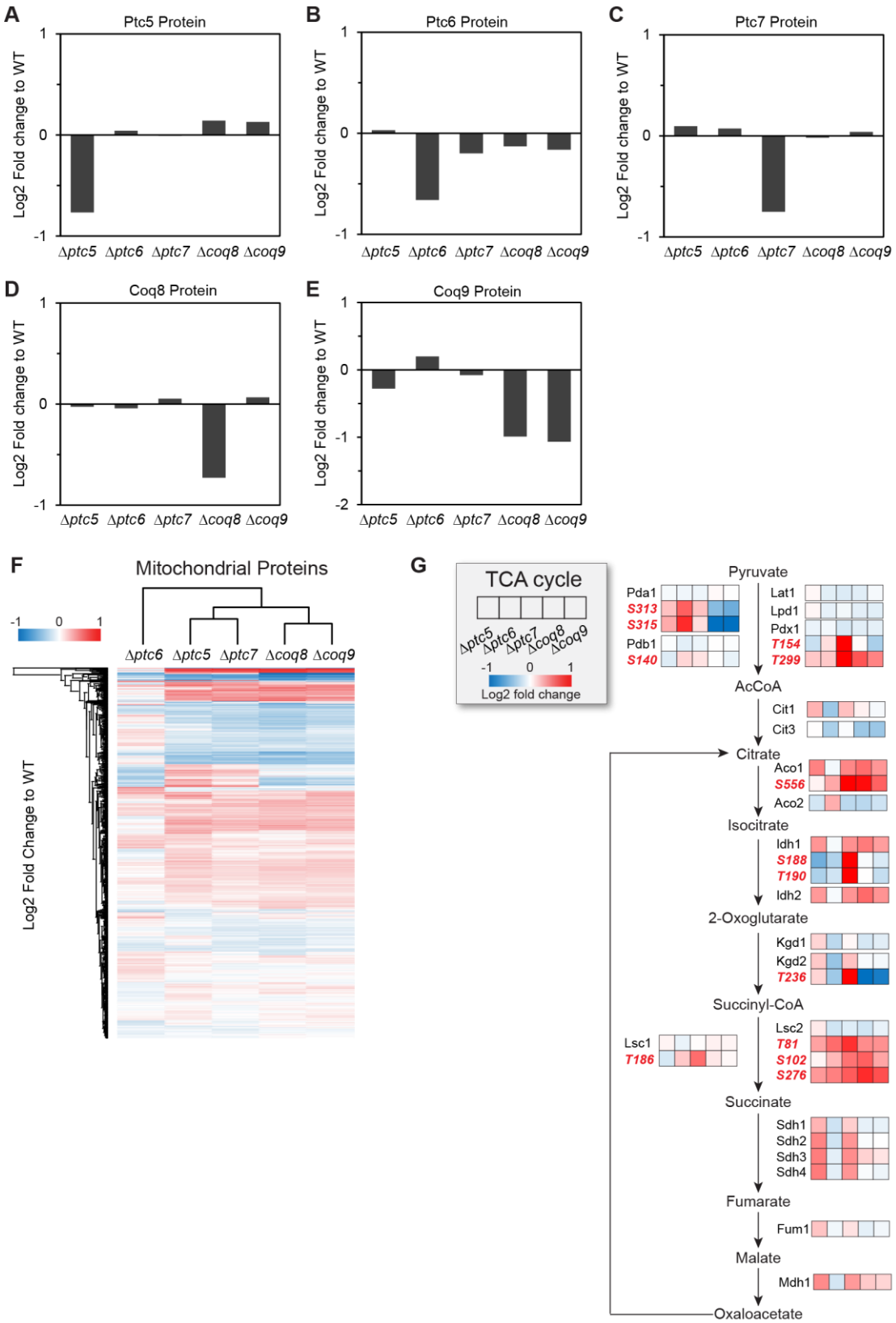

Fig. S2. Analyses of proteome and phosphoproteome. (A-E) Fold changes of (A) Ptc5p, (B) Ptc6p, (C) Ptc7p, (D) Coq8p, and (E) Coq9p proteins quantified by mass spectrometry (MS) from Δ*ptc5*, Δ*ptc6*, Δ*ptc7*,

Δ*coq8*, and Δ*coq9*. (F) Hierarchical clusters of Δ*gene* yeast strains and all quantified mitochondrial proteins. (G) Heat maps of fold changes of proteins and phosphoisoforms from the tricarboxylic acid (TCA) cycle as quantified by MS. Black symbols represent protein; red symbols represent phosphoisoforms.

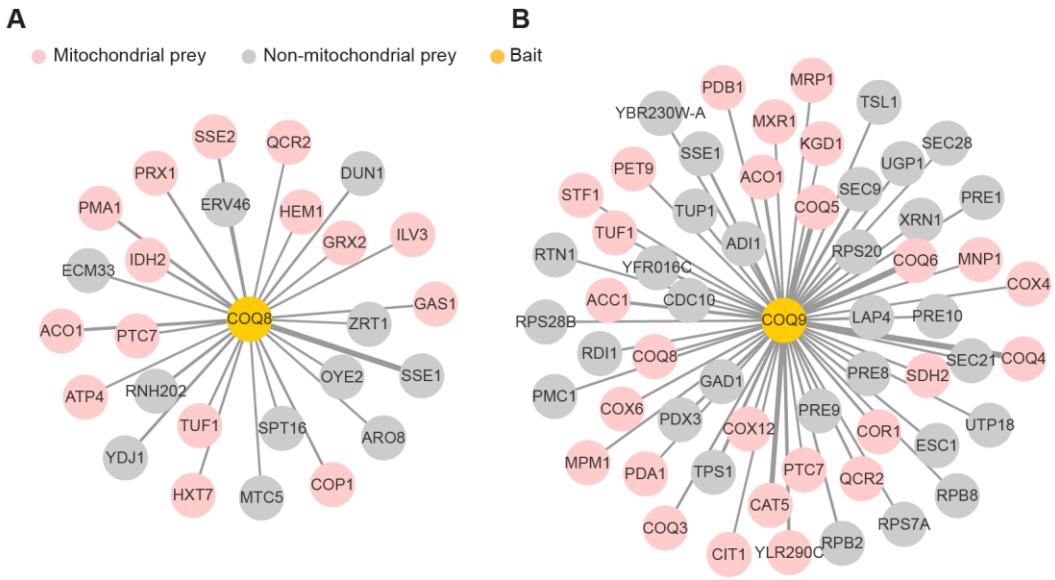

Fig. S3. Interaction networks of Coq8p and Coq9p. (A-B) Interaction networks of (A) Coq8p and (B) Coq9p. Pink circles represent mitochondrial protein interactors; grey circles represent non-mitochondrial protein interactors; yellow circles represent baits. Line width correlates with the number of PSMs identified for each interactor.

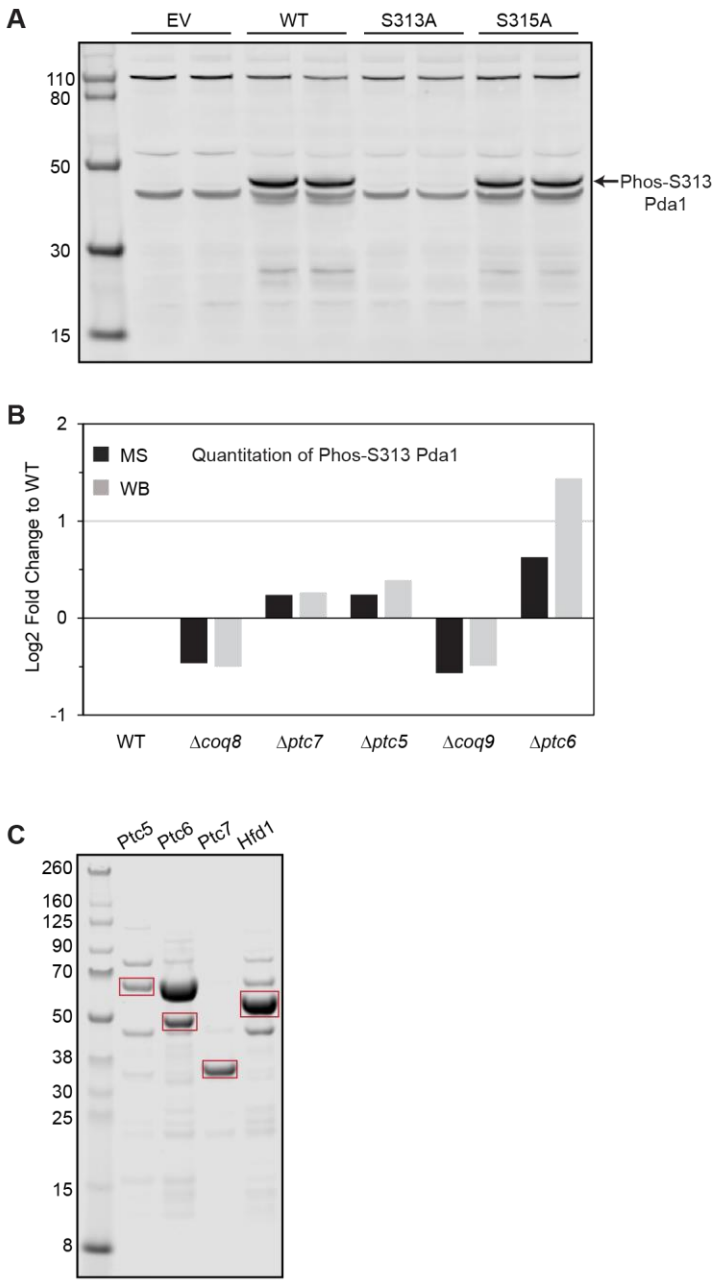

Fig. S4. *In vitro* biochemical assays. (A) Immunoblot against phosphorylated human PDHA1 (homolog of Pda1p) on S264. Whole cell lysates from Δ*pda1* yeast expressing empty vector (EV), wild-type (WT), S313A, and S315A *pda1*. The arrow identifies the band specific for phosphorylated Pda1p on S313. The left lane is protein ladder with molecular weight (kDa) labeled on the side. (B) Fold change of phosphorylated Pda1p on S313 quantified by MS (black bar) or western blotting (WB, grey bar) from WT, Δ*coq8*, Δ*ptc7*, Δ*ptc5*, Δ*coq9*, and Δ*ptc6.* (C) Coomassie staining of recombinant Ptc5p, Ptc6p, Ptc7p, and Hfd1p. Red boxes circle the bands for recombinant proteins. The left lane is protein ladder with molecular weight (kDa) labeled on the side.

OTHER SUPPLEMENTAL FILES

Table S1 (Microsoft Excel format). Phosphoproteomic Data
Table S2 (Microsoft Excel format). Proteomic Data
Table S3 (Microsoft Excel format). Protein Interaction Data