

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

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Running Title: *Ptc6p is the yeast pyruvate dehydrogenase phosphatase*

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## ABSTRACT

The pyruvate dehydrogenase complex (PDC) is the primary metabolic checkpoint connecting glycolysis and mitochondrial oxidative phosphorylation, and is important for maintaining cellular and organismal glucose homeostasis. Phosphorylation of the PDC E1 subunit was identified as a key inhibitory modification in bovine tissue approximately 50 years ago, and this regulatory process is now known to be conserved throughout evolution. Although *Saccharomyces cerevisiae* is a pervasive model organism for investigating cellular metabolism and its regulation by signaling processes, the phosphatase(s) responsible for activating the PDC in *S. cerevisiae* has not been conclusively defined. Here, using comparative mitochondrial phosphoproteomics, analyses of protein-protein interactions by affinity enrichment-mass spectrometry, and *in vitro* biochemistry, we define Ptc6p as the primary PDC phosphatase in *S. cerevisiae*. Our analyses further suggest additional substrates for related *S. cerevisiae* phosphatases and describe the overall phosphoproteomic changes that accompany mitochondrial respiratory dysfunction. In summary, our quantitative proteomics and biochemical analyses have identified Ptc6p as the primary—and likely sole—*S. cerevisiae* PDC phosphatase, closing a key knowledge gap about

the regulation of yeast mitochondrial metabolism. Our findings highlight the power of integrative omics and biochemical analyses for annotating the functions of poorly characterized signaling proteins.

Cells must to adapt to frequently changing nutritional and environmental conditions in order to maintain metabolic homeostasis. Post-translational modifications (PTMs) are essential cellular “tools” for enabling this metabolic adaptation across short- and long-term time scales. Recent studies have revealed that mitochondrial proteins are replete with PTMs, including phosphorylation (1), acetylation (2), and nitrosylation (3), suggesting that these modifications may play prominent roles in regulating mitochondrial activities. However, it has been challenging to distinguish mitochondrial PTMs that occur in a non-enzymatic fashion, such as certain autophosphorylation and acylation reactions (4,5), from those that are regulated enzymatically. Moreover, for the latter case, it has proven difficult to pinpoint the specific enzymes (e.g., kinases and phosphatases) responsible for this regulation.

The first example of a regulatory mitochondrial PTM, discovered around 50 years ago (6), is the reversible phosphorylation on the E1 $\alpha$  subunit of the bovine pyruvate dehydrogenase

complex (PDC)—the three-enzyme complex in the mitochondrial matrix that converts pyruvate into acetyl-CoA, thereby linking glycolysis to mitochondrial functions including the tricarboxylic acid (TCA) cycle, mitochondrial respiration, and fatty acid biosynthesis. The mammalian PDC has three phosphosites (7) that are collectively regulated by four kinase isoenzymes (PDK1-4) (8,9) and two phosphatase isoforms (PDP1-2) (10) that differ in their tissue distribution and regulatory characteristics (11). Tracking the altered expression of these enzymes has lent important insight into how and when PDC function is regulated or dysregulated, such as the increased expression of PDK1 during hypoxia (12,13), the suppression of PDK1 expression accompanied with induction of PDP2 in oncogene-induced senescence (14), and the decreased expression of PDP1 during cardiomyocyte differentiation (15) and in obesity (16).

*Saccharomyces cerevisiae* is a prominent model organism for diverse areas of biological research. Thanks to its genetic tractability, short generation time, conserved genome, and to the vast arsenal of available biological research tools, *S. cerevisiae* has emerged as a dependable model system even for complex human diseases and processes (17), including neurodegeneration and aging (18,19). *S. cerevisiae* has also been the focus of major efforts to reconstruct a comprehensive cellular metabolic network (20), and to explore the importance of PTMs in regulating metabolism (21). Despite this, our understanding of PDC regulation in *S. cerevisiae* surprisingly lags behind that of the mammalian PDC. In particular, despite significant recent progress (22,23), the identity of the *S. cerevisiae* PDC phosphatase(s) has not been fully established.

Here, we performed integrative proteomics and biochemical analyses to define Ptc6p as the primary PDC phosphatase. Our data also suggest potential direct targets/physiological roles for Ptc5p and Ptc7p, and reveal the dynamic mitochondrial protein phosphorylation changes that accompany respiratory dysfunction in *S. cerevisiae*.

## RESULTS

**Primary Sequence Analysis of PDPs and *S. cerevisiae* PP2Cs**—The mammalian PDC has three phosphosites on its E1 $\alpha$  subunit: S264, S271, and S203 (corresponding to the mature human

sequence), all of which are inhibitory (7,24,25). One of these sites—S264, which has the highest phosphorylation occupancy and is phosphorylated most rapidly (7,26)—is conserved in *S. cerevisiae* (S313). The kinases responsible for the phosphorylation of these residues in mammals (PDK1-4) (8,9) and in *S. cerevisiae* (Pkp1p,2p) (22,23) have been defined, as have the phosphatases in the mammalian system (PDP1,2) (10). In contrast, the *S. cerevisiae* phosphatase(s) is not fully established (Fig. 1).

Given the overall conservation of the PDC regulatory system between mammals and *S. cerevisiae*, we first attempted to identify the most likely candidate(s) for the *S. cerevisiae* PDC phosphatase(s) via primary sequence analyses. The mammalian PDC phosphatases belong to the metal-dependent serine/threonine protein phosphatase 2C (PP2C) family. *S. cerevisiae* has seven PP2C family members (Ptc1p-7p), three of which have been localized to mitochondria (Fig. 2A). A phylogenetic analysis of *S. cerevisiae* PP2C phosphatases (Fig. 2A, S1) with PDPs from diverse species did not reveal a clear PDP ortholog. The three *S. cerevisiae* mitochondrial phosphatases were more closely related to human PDP1 or PDP2 than were the others, with Ptc5p and Ptc6p having the highest homology. However, these phosphatases share only modest sequence identity with each other and with human PDP1 or PDP2 (Fig. 2B). Importantly, both Ptc5p and Ptc6p have been implicated as PDC phosphatases in *S. cerevisiae* (22,23); however, whether either or both of these phosphatases act directly on the PDC, or instead affect PDC activity indirectly, remains unclear. As such, the *bona fide* PDC phosphatase(s) has not been conclusively defined, and cannot be established confidently via primary sequence analyses.

**Comparative Mitochondrial Phosphoproteomic Analyses in  $\Delta$ ptc Yeast Strains**—To begin identifying potential *S. cerevisiae* PDC phosphatase(s) experimentally, we performed a quantitative phosphoproteomics analysis of wild type (WT) and knockout (KO) strains (Fig. 3A, Table S1, S2). We focused on strains lacking Ptc5p, Ptc6p, and Ptc7p due to their mitochondrial localization (22,27,28). Additionally, because deletion of each of these phosphatases causes varying levels of respiratory dysfunction, we analyzed strains lacking Coq8p and Coq9p—proteins essential for mitochondrial

coenzyme Q (CoQ) production, and thus respiratory competency—as controls (29,30). These experiments were designed to identify potential substrates, including Pda1p (the *S. cerevisiae* PDC E1 $\alpha$  subunit), for three mitochondrial phosphatases, and to assess the overall phosphoproteomics changes that accompany general mitochondrial respiratory incompetency.

All yeast strains were grown in rich media containing 2% glucose (w/v) until 4 hours past the diauxic shift. At this point, yeast transition from a fermentative metabolism to mitochondria-dependent respiratory metabolism. A crude mitochondrial enrichment was performed, and 6-plex tandem mass tags (TMT) were used for proteomic and phosphoproteomic quantification. Phosphoisoform fold changes were normalized to the corresponding protein fold change. Overall, we quantified 3184 proteins, including 823 mitochondrial proteins (annotated using the Yeast MitoMiner database (31)), and 2335 phosphoisoforms (267 mitochondrial) (Table S1, S2). All KO strains exhibited the expected abundance decrease of the proteins encoded by the deleted genes (Fig. S2A-E). Unsupervised hierarchical clustering of the fold changes for both mitochondrial phosphoisoforms and proteins showed distinct profiles for each phosphatase KO strain (Fig. 3B, S2F). Consistent with their shared function, strains lacking Coq8p and Coq9p generated similar proteomic and phosphoproteomic profiles (Fig. 3B, S2F). Additionally, the abundance of Coq9p was diminished in the  $\Delta coq8$  strain (Fig. S2E), consistent with previous reports that these proteins exist in a biosynthesis complex (complex Q) (32,33). Together, these data argue against Ptc5p, Ptc6p, or Ptc7p possessing largely redundant functions, as is the case for mammalian PDP1 and PDP2.

To identify potential direct substrates for each phosphatase, we analyzed the individual phosphoproteomic changes in each strain, with the hypothesis that deletion of a phosphatase would cause an increased phosphorylation level of its substrate(s). Consistent with prior studies (34), we used a fold change cutoff of 1.5 (50%; log<sub>2</sub> fold change of 0.585) to define increased phosphorylation. Based on this criterion, and filtering for proteins in the Yeast MitoMiner database, Ptc5p, Ptc6p, and Ptc7p had 25, 5, and 68

hits, respectively (Fig. 3C-E, Table S1). Surprisingly, despite having the fewest overall changes, only the  $\Delta ptc6$  strain exhibited a marked increase in Pda1p S313 phosphorylation (Fig. 3D).

Based on our phosphoproteome data, we propose that Ptc5p possesses *in vivo* function(s) distinct from direct PDC regulation. For example, phosphorylation of S25 and/or S27, and S24 on Gpd1p are increased in  $\Delta ptc5$  yeast (Fig. 3C). Gpd1p is one of the two glycerol-3-phosphate dehydrogenase isoforms in *S. cerevisiae* (35), and its activity is regulated by the kinases Ypk1p and Ypk2p (36); however, the corresponding phosphatase(s) remains unknown. Gpd1p is found in the cytosol and peroxisomes (37), but has been localized to mitochondria by high-throughput studies (38). Thus, it is possible that Ptc5p is responsible for dephosphorylating the mitochondrial fraction of Gpd1p.

Of course, it is likely that some elevated phosphoisoforms across our dataset represent downstream compensatory changes instead of being direct targets of these phosphatases, such as T31 on Gph1p in  $\Delta ptc6$  (Fig. 3D). Gph1p is the *S. cerevisiae* glycogen phosphorylase, which catalyzes the breakdown of glycogen into glucose subunits for glycolysis in the cytoplasm, and is activated by phosphorylation of T31 (39). In  $\Delta ptc6$  yeast, the enhanced phosphorylation of Pda1p could slow entry of pyruvate-derived acetyl-CoA into the TCA cycle, thereby causing a compensatory increase in glycolytic flux for ATP generation by increasing the phosphorylation of Gph1p. These data lend insight into the specific metabolic adaptations of each KO strain, and demonstrate that additional experiments are needed to distinguish direct substrates from indirect adaptive changes.

**Affinity-Enrichment Mass Spectrometry (AE-MS) Analyses Directly Link Ptc6p to the PDC**—To help distinguish direct phosphatase substrates from general adaptive changes, we next performed AE-MS. AE-MS is an approach to identify protein-protein interactions (PPIs) across multiple “bait” proteins that combines quantitative MS with a scoring algorithm (here, CompPASS (40)) to differentiate between informative interactions and nonspecific background co-enrichment (33).

We began our AE-MS analyses by immunoprecipitating each bait protein (Ptc5p, Ptc6p, Ptc7p, Coq8p, and Coq9p), which had been

modified to include a C-terminal FLAG-tag (Fig. 4A). The resulting protein eluate was analyzed using nanoflow liquid chromatography coupled to high-resolution MS (Q Exactive Hybrid Quadrupole-Orbitrap, nLC-MS/MS). Collectively, analysis of these five baits yielded 118 high-confidence mitochondrial PPIs from amongst 1750 total PPIs identified. These included interactions between our Cop9p control and seven other proteins involved in CoQ biosynthesis (Coq3p, Coq4p, Coq5p, Coq6p, Cat5p (Coq7p), Coq8p, and Coq11p (Ylr290c)) (Fig. S3B, Table S3). These observations are consistent with recent reports that CoQ-related proteins interact to form a biosynthetic complex (complex Q) (32,33), thereby validating the robustness of our approach.

Our data revealed distinct interaction networks for Ptc5p, Ptc6p, and Ptc7p (Fig. 4B-D, Table S3), further suggesting that these phosphatases perform non-overlapping functions *in vivo*. While Ptc5p and Ptc7p each exhibited a fairly large number of mitochondrial PPIs (~30), perhaps suggesting more pervasive roles in mitochondrial phosphoprotein regulation, Ptc6p exhibited a minimal interaction network of only 8 mitochondrial proteins. Strikingly, four of these eight are subunits of the PDC, including Pda1p, Lat1p (dihydrolipoamide acetyltransferase, the PDC E2 subunit), Pdb1p (the E1 $\beta$  subunit), and Pdx1p (an E3-binding protein) (Fig. 4C). No PDC subunits were observed in the larger Ptc5p and Ptc7p networks (Fig. 4B, 4D).

To further home in on potential direct substrates for these phosphatases, we integrated our phosphoproteomics data with our AE-MS data (Fig. 4D). Phosphatases might only exhibit low-energy, fleeting interactions with their substrate(s), which may not be readily detectable by our PPI analysis method. Nonetheless, we reasoned that proteins whose phosphorylation levels increased following KO of a phosphatase, and that also physically interacted with that phosphatase, would likely be direct substrates. Although Ptc6p had very few hits in either category, Pda1p stood out in both analyses (Fig. 4E). Ptc5p had no overlapping targets, while Ptc7p had three, albeit with lower interaction scores than that for Ptc6p-Pda1p (Fig. 4D, Table S3). Collectively, these analyses implicate each phosphatase in diverse mitochondrial activities, and strongly suggest that Pda1p is a direct substrate of Ptc6p.

*In vitro* Biochemical Assays Verify Ptc6p as a Pda1p Phosphatase—We next performed a series of biochemical experiments to validate the results from our large-scale analyses and to further test the hypothesis that Ptc6p is the primary PDC phosphatase. First, using a phospho-specific antibody against Pda1p S313 (Fig. S4A), we performed immunoblots as an orthogonal approach to assess the relative change in this phosphoisoform between WT and each of our five KO strains. Consistent with our MS analyses, the  $\Delta ptc6$  strain exhibited a marked increase in Pda1p S313 phosphorylation, whereas the  $\Delta ptc5$  and  $\Delta ptc7$  strains showed little difference from WT (Fig. 5A, S4A, S4B). Notably, our immunoblotting results suggest an even larger increase in Pda1p S313 phosphorylation (~3 fold change) in  $\Delta ptc6$  yeast than was estimated by our MS analyses (Fig. S4B), potentially due to dynamic range suppression—a known caveat of MS isobaric tagging methods that can cause an underestimation of fold-change values (41).

Next, to further test the interaction between Ptc6p and Pda1p identified by AE-MS, we immunoprecipitated each FLAG-tagged phosphatase and blotted directly for Pda1p. Despite comparable immunoprecipitation efficiencies, Pda1p again only stood out as an interacting protein of Ptc6p (Fig. 5B). These data confirm the *in vivo* physical interaction between Ptc6p and the PDC, likely via its direct interaction with Pda1p (Fig. 5B).

All three phosphatase deletion strains exhibited lower oxygen consumption rates (OCRs) than WT when grown on pyruvate-based media, with  $\Delta ptc6$  less able to utilize pyruvate for oxygen consumption than  $\Delta ptc5$  (Fig. 5C). However, this does not directly reveal the relative contribution of PDC inhibition to each strain's growth deficiency. Phosphorylation of Pda1p is an inhibitory modification (42); as such, the relative changes in Pda1p phosphorylation generally should be anti-correlated with PDC activity. To test whether this indeed holds true across our strains, we measured PDC activity in mitochondrial lysates from WT,  $\Delta ptc5$ ,  $\Delta ptc6$ , and  $\Delta ptc7$  yeast. The  $\Delta ptc6$  strain possessed very low PDC activity (less than 10% compared to WT, Fig. 5D), suggesting that PDC inhibition plays a major role in this strain's compromised oxygen consumption on pyruvate-based media. The  $\Delta ptc5$  and  $\Delta ptc7$  strains also showed a more modest decrease in PDC activity



(~50% of WT). This decrease is not surprising both because the  $\Delta ptc5$  and  $\Delta ptc7$  strains exhibited a slightly increased phosphorylation level of Pda1p S313 by immunoblot or MS analyses (Fig. S4B, Table S1), and because the loss of these phosphatases is known to cause general mitochondrial dysfunction, which can lead to PDC inactivation by other means. Importantly, the protein abundance of Pda1p did not change appreciably across these four strains based on our proteomics analysis (Fig. 5E).

Finally, to test the ability of Ptc5p, Ptc6p, and Ptc7p to dephosphorylate Pda1p more directly, we purified recombinant versions of each phosphatase from *E. coli*, along with Hfd1p (a fatty aldehyde dehydrogenase involved in CoQ biosynthesis that served as a negative control) (43) (Fig. S4C). We then incubated equal amounts of each protein with mitochondrial lysate from  $\Delta ptc6$  (where Pda1p phosphorylation is highest) and analyzed Pda1p S313 phosphorylation at different time points. Only Ptc6p was able to dephosphorylate Pda1p *in vitro* (Fig. 6A), despite the fact that all three purified phosphatases were able to dephosphorylate *para*-nitrophenyl phosphate (pNPP), albeit with differing rates (Fig. 6B). Last, we measured the PDC activity in these lysates at the 30 minute incubation time point and found, consistently, that the Ptc6p-treated lysates clearly had the highest PDC activity (Fig. 6C).

Collectively, our integration of quantitative MS-based comparative phosphoproteomics with AE-MS assessment of PPIs and *in vitro* biochemical analyses reveals that Ptc6p is the primary—and likely sole—*S. cerevisiae* PDC phosphatase. Our data point to distinct, non-overlapping functions for the closely-related mitochondrial phosphatases, Ptc5p and Ptc7p. Finally, our work provides a prioritized list of direct substrates for each phosphatase, and describes the overall phosphoproteomic changes that accompany mitochondrial respiratory dysfunction.

## DISCUSSION

Metabolic processes are under exquisite control by signaling pathways that use PTMs, such as phosphorylation, to alter the function of target proteins. However, defining how a given signaling pathway imparts such post-transcriptional control of a metabolic event is difficult, and demands that at least three parameters be established: 1) the

identification of the PTMs themselves, 2) the determination of the functional consequence(s) of the modification(s), and 3) the identification of the regulatory enzymes (e.g., kinases and phosphatases) that control the PTM abundance. Recent advances in MS-based analyses have rapidly accelerated the first of these challenges (44), but the latter two remain difficult and laborious.

The mammalian mitochondrial PDC is a classic metabolic target of reversible phosphorylation. In this case, the phosphosites (S264, S271, and S203) (7,24), the regulatory effect (inhibition) (25), and the regulatory machinery (PDK1-4; PDP1,2) (8-10) have all been established. Control of PDC activity is essential for regulating the entry of glycolysis-derived pyruvate into the TCA cycle, and the overall maintenance of cellular and organismal glucose homeostasis. Dysfunction of the core PDC or the phosphatases that regulate it leads to pyruvate dehydrogenase deficiency (45,46). Additionally, dichloroacetate (DCA), a small molecule inhibitor of PDK, has been explored as a potential therapy for cancer (47). More broadly, understanding when PDC kinases and phosphatases are induced or repressed lends important insight into the metabolic state of a cellular system.

*S. cerevisiae* has emerged as an important model organism for studying basic metabolism and its regulation by signaling processes, including the PDC system. Despite this, surprisingly, the identity of the *S. cerevisiae* PDC phosphatase has not been clearly established. Recent work by Krause-Buchholz *et al.* linked two of the *S. cerevisiae* PP2C-type phosphatases, Ptc5p and Ptc6p, to PDC function (22,23). In these studies, the authors demonstrated that loss of either enzyme resulted in decreased PDC activity concomitant with elevated PDC phosphorylation, and that each phosphatase potentially associated with Pda1p. However, direct evidence of their ability to dephosphorylate PDC and their physical interaction with Pda1p were lacking, and the sequence divergence between these phosphatases suggests that they are unlikely to perform redundant functions. As such, we decided to revisit whether one of these phosphatases—or a distinct, but related phosphatase—serves as the primary PDC phosphatase.

Through a combination of comparative phosphoproteomics, AE-MS, and *in vitro* biochemistry, we demonstrated that Ptc6p is the

primary PDC phosphatase in *S. cerevisiae*, and suggest orthogonal functions for the related PP2C phosphatases, Ptc5p and Ptc7p. Ptc5p exhibited a distinct set of potential substrates, including a possible role in regulating mitochondrial Gpd1p. Unique substrates likewise emerged for Ptc7p, including Yml6p, which was a top substrate candidate in our recent analysis (48). Notably, some different potential substrates for Ptc7p were identified between this study and our previous study (48), in which the growth conditions of the yeast were different. This suggests that it is still possible for Ptc5p to work directly on the PDC under a different metabolic state. However, given our data, we believe that a more auxiliary role for Ptc5p in PDC function—such as in complex assembly, as previously suggested (23)—is more likely.

Overall, our quantitative proteomics and biochemical analyses have built upon recent work (22) to help establish Ptc6p as the *S. cerevisiae* PDC phosphatase, thereby filling a key gap in knowledge regarding the regulation of yeast mitochondrial metabolism. More generally, our work reveals the power of integrative omics and biochemical analyses for annotating the functions of poorly characterized signaling proteins.

## EXPERIMENTAL PROCEDURES

**Yeast Strain used in this Study**—WT,  $\Delta ptc5$ ,  $\Delta ptc6$ ,  $\Delta ptc7$ ,  $\Delta coq8$ , and  $\Delta coq9$  BY4741 yeast (MATa *his3 $\Delta$ 1 leu2 $\Delta$ 0 met15 $\Delta$ 0 ura3 $\Delta$ 0*) were purchased from Open Biosystems. The identity of each KO strain was verified by PCR. The endogenously three tandem hemagglutinin (3xHA) tagged *pda1* strain was made as previously described (49).

**Quantitative Proteomics and Phosphoproteomics Analyses**—Briefly, crude mitochondria were lysed, digested, TMT labeled, and fractionated by strong cation exchange (SCX) chromatography (1). Phosphopeptide enrichment was done with immobilized metal affinity chromatography (IMAC). 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). A customized software Coon OMSSA Proteomics Software Suite (COMPASS) was used to analyze the MS raw data.

**Protein Interaction Study**—C-terminal

FLAG tagged protein constructs in p416 GPD plasmid were transformed into WT BY4741 yeast. Yeast cells were harvested and lysed. The clarified supernatant was incubated with anti-FLAG M2 magnetic beads (Sigma) followed with five washes and eluted with FLAG peptide. The samples were subjected to MS analysis on a Q Exactive hybrid Quadrupole-Orbitrap mass spectrometer (Thermo). CompPASS (WD) scoring was used to filter interaction partners (33,40). For immunoblot verification of the Ptc6p and Pda1p interaction, an endogenously 3xHA tagged *pda1* strain was transformed with an empty vector or C-terminal FLAG tagged protein construct (*ptc5*, *ptc6*, *ptc7*, *coq8*, and *coq9*) in p416 GPD plasmid. Clarified cell lysate (input) and final elution were collected and used for immunoblotting.

**PDC Activity Assay**—Purified mitochondria were lysed, clarified, and quantified via Bicinchoninic acid (BCA) assay. The PDC activity assay was done in a 96-well plate containing 50  $\mu$ L reaction buffer (150 mM MOPS, 3 mM MgCl<sub>2</sub>, 0.03 mM CaCl<sub>2</sub>, pH 8.0), 20  $\mu$ L of 3 mM thiamine pyrophosphate (TPP), 20  $\mu$ L of 25 mM  $\beta$ -Nicotinamide adenine dinucleotide sodium salt (NAD<sup>+</sup>), 20  $\mu$ L of 38mM cysteine (pH 7.4), 20  $\mu$ L of 50 mM sodium pyruvate, and 50  $\mu$ L of 0.5g/L mitochondrial lysate (50). 20  $\mu$ L of 1.5 mM Coenzyme A sodium salt was added just before starting the assay. A340nm was monitored to calculate nicotinamide adenine dinucleotide (NADH) production rate.

**In vitro Phosphatase Assays**—*In vitro* pNPP phosphatase assays were done in a 96-well plate. 30  $\mu$ L of 50 ng/ $\mu$ L protein was added to 150  $\mu$ L reaction buffer (66.7 mM Tris, 13.3 mM MnCl<sub>2</sub>, pH 8.0). The reaction was initiated by addition of 20  $\mu$ L of 500 mM pNPP, and A405nm was recorded over time. The initial slope was calculated to represent the reaction rate. The relative phosphatase activity is normalized to total protein amount. For *in vitro* phosphatase assays on Pda1p, purified mitochondria from  $\Delta ptc6$  yeast were lysed. 200  $\mu$ L of 100 mM MnCl<sub>2</sub> and 0.2  $\mu$ L of 100 mM CaCl<sub>2</sub> were added into each aliquot of clarified supernatant before addition of 60  $\mu$ L of 50 ng/ $\mu$ L recombinant protein. The mixture was incubated at room temperature. 20  $\mu$ L sample was taken out at different time points, mixed with 4 x sample buffer, and boiled at 95°C for 5 min before western blotting. A separate aliquot of 10  $\mu$ L sample after

30 min treatment was used for the PDC activity assay.

**Protein Alignment and Generation of Phylogenetic Tree**—Protein sequences were downloaded from the Uniprot (<http://www.uniprot.org/>) or NCBI (<http://www.ncbi.nlm.nih.gov/>). Protein alignment

and phylogeny were done using MacVector. ClustalW algorithm with default settings was chosen for protein alignment.

**Statistics**—p values were calculated by a two-tailed Student's t test.

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**Author contributions:** XG and DJP conceived of the project and its design, analyzed data, and wrote the manuscript. XG and NMN performed all experiments. JJC provided key resources and experimental infrastructure.

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## FOOTNOTES

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The abbreviations used are: PDC, pyruvate dehydrogenase complex; PPI, protein-protein interaction; PTM, post-translational modification; TCA, tricarboxylic acid; PP2C, protein phosphatase 2C; WT, wild type; KO, knockout; CoQ, coenzyme Q; TMT, tandem mass tags; AE-MS, affinity-enrichment mass spectrometry; pNPP, *para*-nitrophenyl phosphate.

## FIGURE LEGENDS

**FIGURE 1.** Current model of PDC regulation by reversible phosphorylation. Activity of the PDC is regulated by reversible phosphorylation, but the phosphatase that dephosphorylates the *S. cerevisiae* PDC remains unclear.

**FIGURE 2.** Primary sequence analysis of PDPs and *S. cerevisiae* PP2Cs. (A) Phylogenetic tree of vertebrate PDP1 and PDP2, human PPM1A, and seven *S. cerevisiae* PP2C phosphatases. Blue region highlights homologs of PDP2; green region highlights homologs of PDP1. (B) Chart showing the percentage similarity and identity between human PPM1A, PDP1, PDP2 and seven *S. cerevisiae* PP2C phosphatases.

**FIGURE 3.** Comparative mitochondrial phosphoproteomic analyses of  $\Delta$ *ptc* yeast strains. (A) Experimental phosphoproteomics workflow. Crude mitochondria from six yeast strains (WT,  $\Delta$ *ptc5*,  $\Delta$ *ptc6*,  $\Delta$ *ptc7*,  $\Delta$ *coq8*, and  $\Delta$ *coq9*) were used. Peptides were tagged with 6-plex TMT for MS-based quantification. The red and white tags in the Phospho Enrichment panel represent the reporter and balancing groups of the TMT tags, respectively. (B) Hierarchical clusters of  $\Delta$ *gene* yeast strains and all quantified mitochondrial phosphoisoforms. (C-E) Fold changes of mitochondrial phosphoisoforms with values larger than 1.5 ( $\log_2$  ( $\Delta$ *gene*/WT)) from (C)  $\Delta$ *ptc5*, (D)  $\Delta$ *ptc6*, and (E)  $\Delta$ *ptc7*. Red arrowheads in (D) denote Pdalp phosphoisoforms.

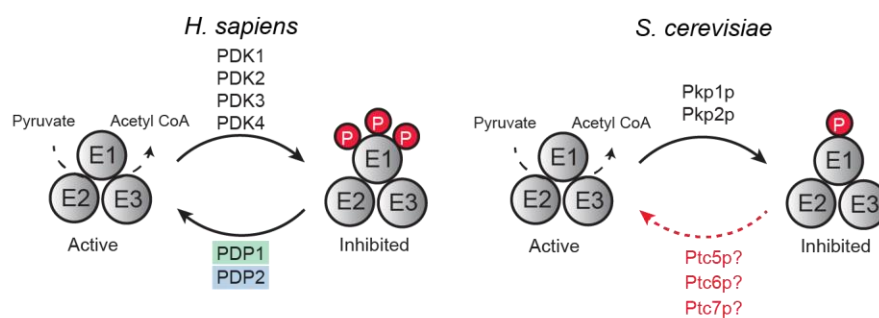
**FIGURE 4.** AE-MS analyses directly link Ptc6p to the PDC. (A) Experimental workflow for AE-MS

analyses. Six baits were used: empty vector (EV), Ptc5p, Ptc6p, Ptc7p, Coq8p, and Coq9p. Eluates from immunoprecipitation were analyzed by LC-MS/MS, and interactions were scored using the CompPASS (WD) scoring algorithm. (B-D) Mitochondrial interaction networks of (B) Ptc5p, (C) Ptc6p, and (D) Ptc7p. Only mitochondrial protein interactors are shown here. Line width correlates with the number of PSMs identified for each interactor. Shading in (C) indicates the Ptc6p interactions with PDC-related proteins. (E) Overlapping hits of mitochondrial interactors and mitochondrial proteins with phosphoisoforms that increased by  $\geq 1.5$ -fold.

**FIGURE 5.** *In vitro* biochemical assays verify Ptc6p as a Pda1p phosphatase. (A) Immunoblot of phosphorylated Pda1p on S313, or VDAC (loading control). Samples are mitochondrial lysates from WT,  $\Delta coq8$ ,  $\Delta ptc7$ ,  $\Delta ptc5$ ,  $\Delta coq9$ , and  $\Delta ptc6$  yeast. (B) Immunoblot of HA (Pda1p was endogenously tagged with HA), or FLAG (bait proteins were C-terminally FLAG-tagged). Samples in the left panels are input lysates for the anti-FLAG immunoprecipitations from WT yeast strains overexpressing EV, Ptc5p, Ptc6p, and Ptc7p. Samples in the right panels are the corresponding eluates from the same immunoprecipitations. (C) Oxygen consumption rates (OCRs) of WT,  $\Delta ptc5$ ,  $\Delta ptc6$ ,  $\Delta ptc7$ , and  $\Delta pda1$  in synthetic complete media containing 2% pyruvate (mean  $\pm$  SD, n=6). (D) Relative PDC activity of mitochondrial lysates from WT,  $\Delta ptc5$ ,  $\Delta ptc6$ , and  $\Delta ptc7$  (mean  $\pm$  SD, n=3). (E) Fold change of Pda1p protein quantified by MS from same samples in (D) (normalized to WT). (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001).

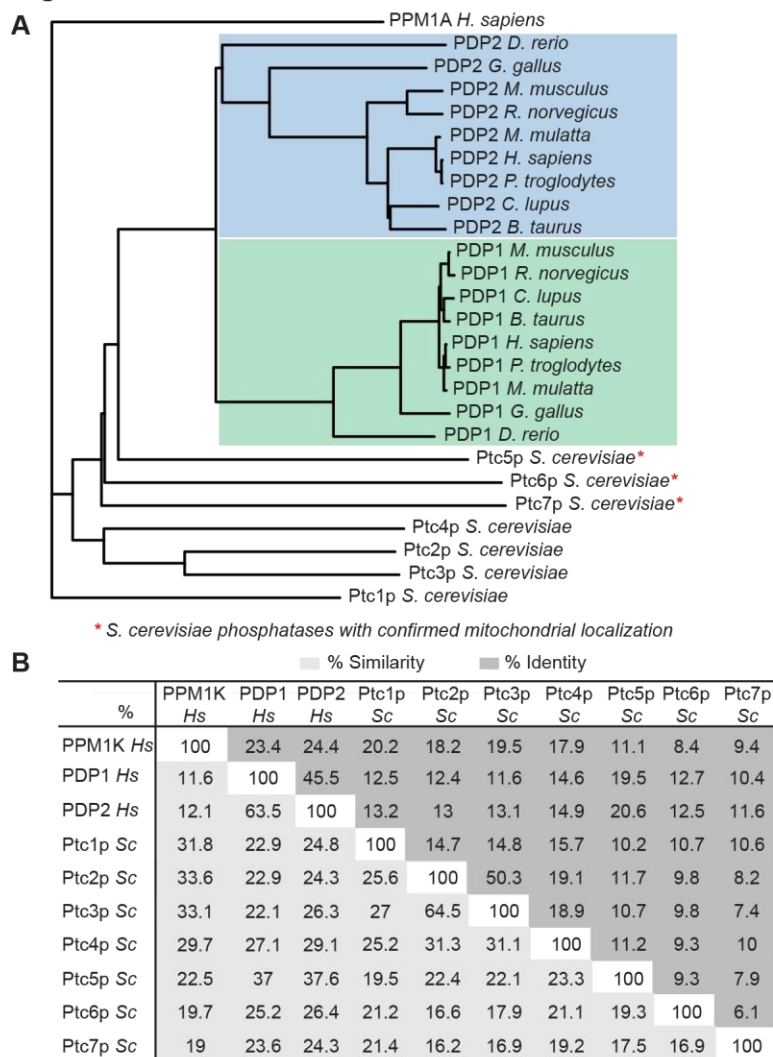
**FIGURE 6.** Only Ptc6p dephosphorylates and activates Pda1p *in vitro*. (A) Immunoblots of phosphorylated Pda1p on S313 (left panels), or VDAC (loading control, right panels). Mitochondrial lysate from  $\Delta ptc6$  was treated with recombinant Ptc5p, Ptc6p, Ptc7p, or Hfd1p. Samples from six different time points were taken and analyzed by immunoblotting. (B) Relative *in vitro* phosphatase activity against pNPP for recombinant Ptc5p, Ptc6p, Ptc7p, or Hfd1p (mean  $\pm$  SD, n=3). The activity is normalized to total protein amount. (C) Relative PDC activity of mitochondrial lysates from  $\Delta ptc6$  yeast after 30 min treatment with recombinant Ptc5p, Ptc6p, Ptc7p, or Hfd1p (mean  $\pm$  SD, n=3). (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001).

**Figure 1**

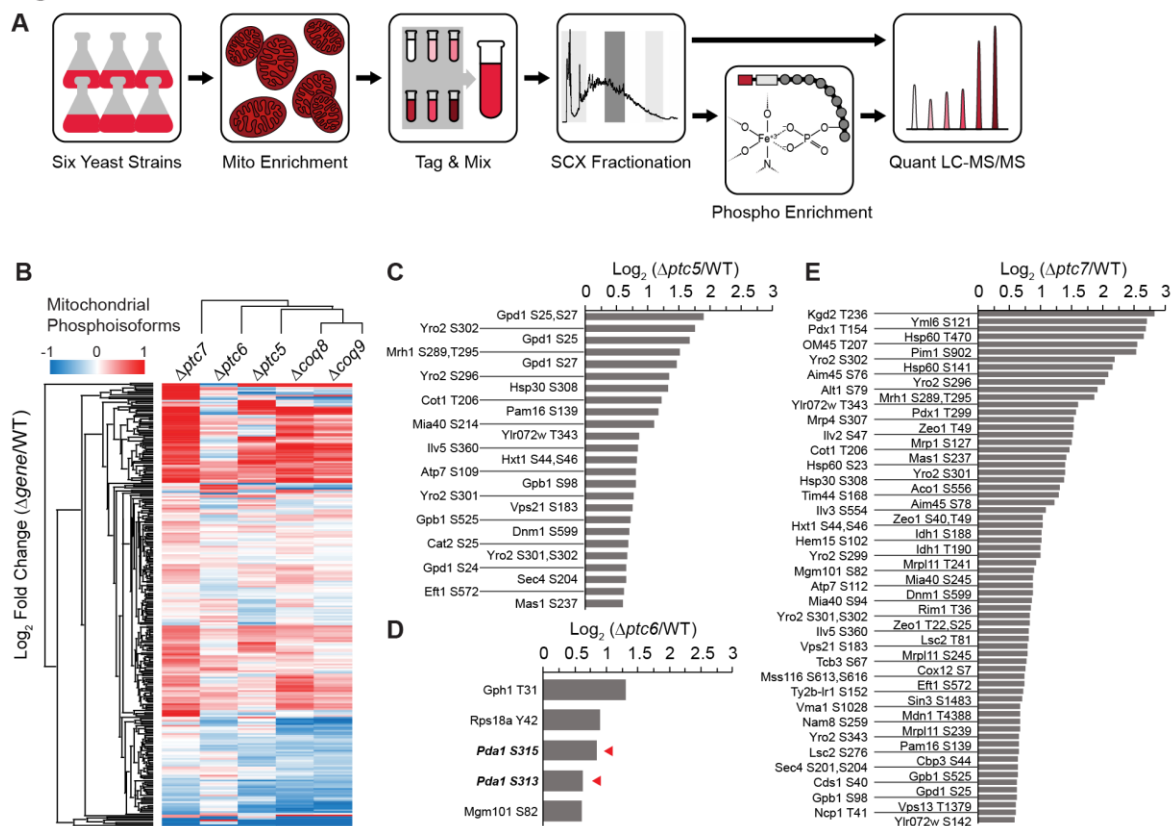




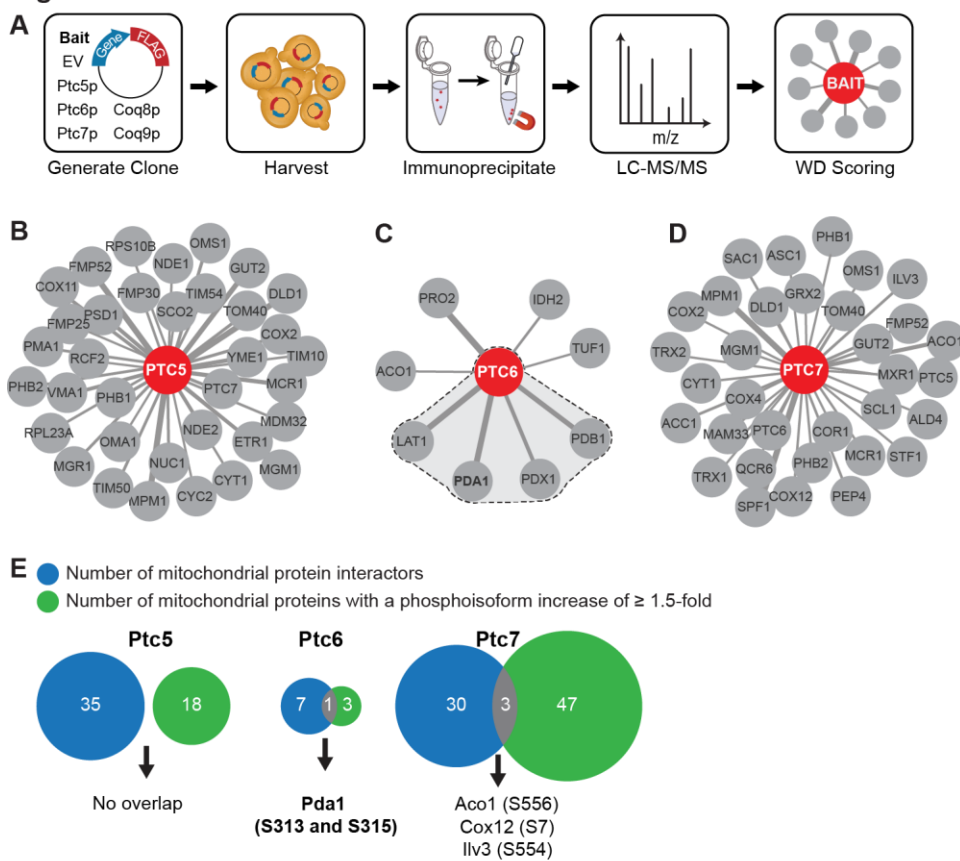
**Figure 2**



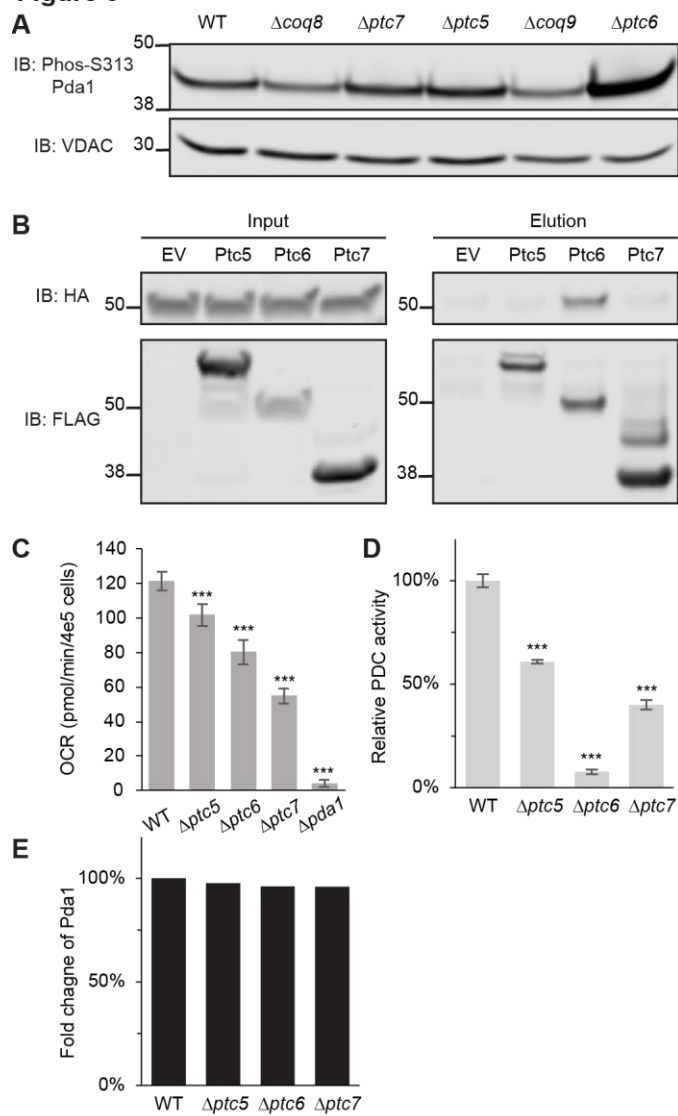
**Figure 3**



**Figure 4**

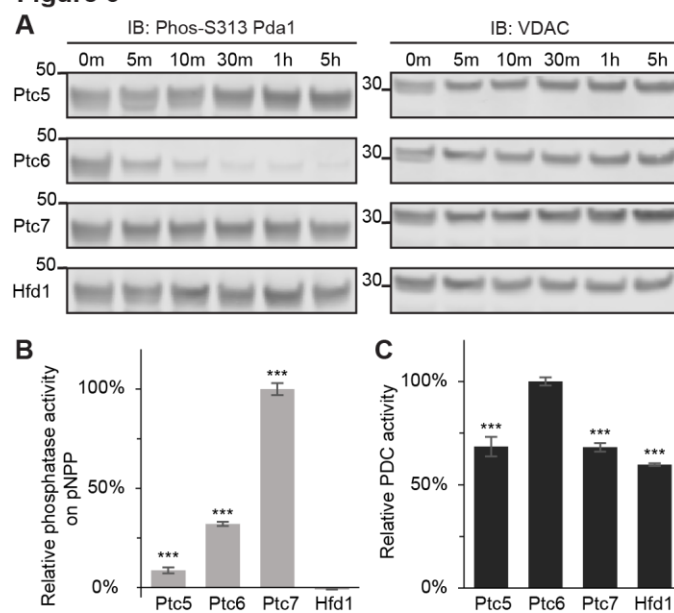


**Figure 5**





**Figure 6**



**Integrative proteomics and biochemical analyses define Ptc6p as the *Saccharomyces cerevisiae* pyruvate dehydrogenase phosphatase**

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