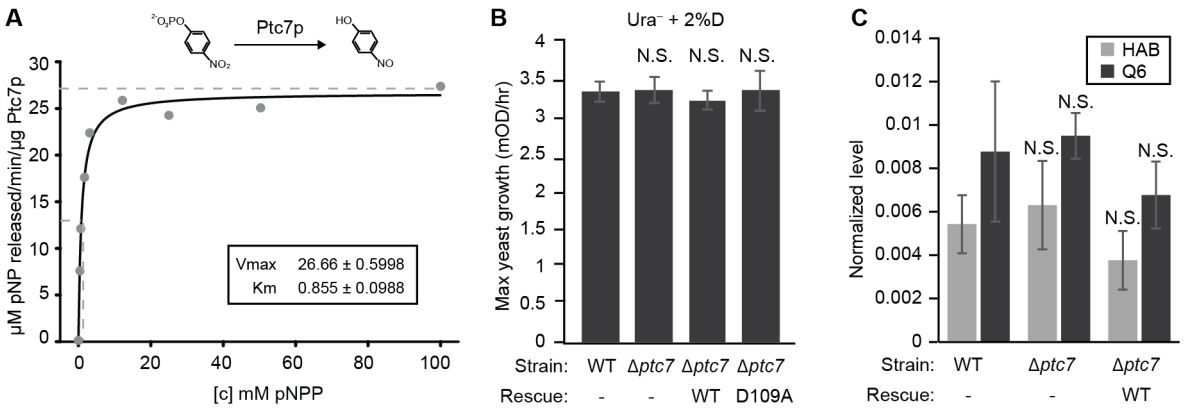

Figure S1, Related to Figure 1. Ptc7p is an active phosphatase *in vitro*. Δ*ptc7* has normal growth rate in dextrose and normal coenzyme Q6 level.

(A) Kinetic curve of *in vitro* phosphatase assay of recombinant Ptc7p on para-nitrophenyl phosphate (pNPP). The inset shows V_{max} and K_m values.

(B) Maximum growth rate of WT and Δ*ptc7* in Ura⁻ synthetic media containing 2% dextrose (mean ± SD, n=10). Rescue indicates if the strain is expressing a plasmid containing Ptc7p (WT: wild type Ptc7p; D109A: catalytically inactive mutant of Ptc7p).

(C) Normalized level of 3-hexaprenyl-4-aminobenzoic acid (HAB), and coenzyme Q6 (Q6) in three strains (WT, Δ*ptc7*, and Δ*ptc7* expressing WT Ptc7p, mean ± SD, n=3).

* p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; N.S., not significant.

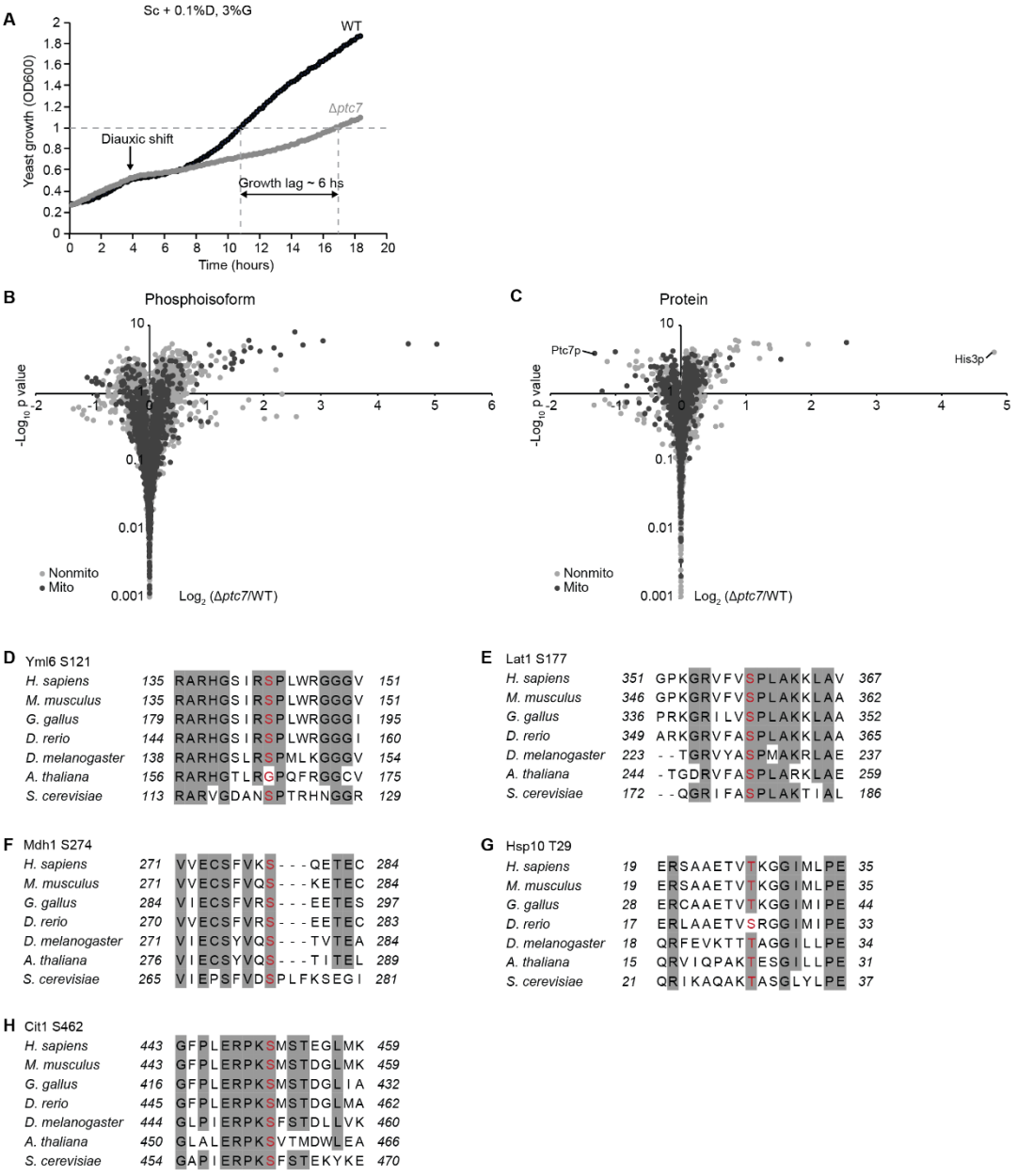

Figure S2, Related to Figure 2. Proteomics and phosphoproteomics results.
(A) Growth curve of WT and Δ*ptc7* in synthetic complete (SC) media with 0.1% dextrose and 3% glycerol. Diauxic shift and growth lag are indicated on the plot.
(B) Fold changes in phosphoisoforms abundances ($\log_2$(Δ*ptc7*/WT), n=3) versus statistical significance (-$\log_{10}$(p-value)). Both mitochondrial (mito) and non-mitochondrial (nonmito) ones are plotted here.
(C) Fold changes in protein abundances ($\log_2$(Δ*ptc7*/WT), n=3) versus statistical significance (-$\log_{10}$(p-value)). Ptc7p and His3p are highlighted.
(D) Homolog sequence alignments of Yml6, (E) Lat1, (F) Mdh1, (G) Hsp10, and (H) Cit1. Phosphosites are highlighted in red.

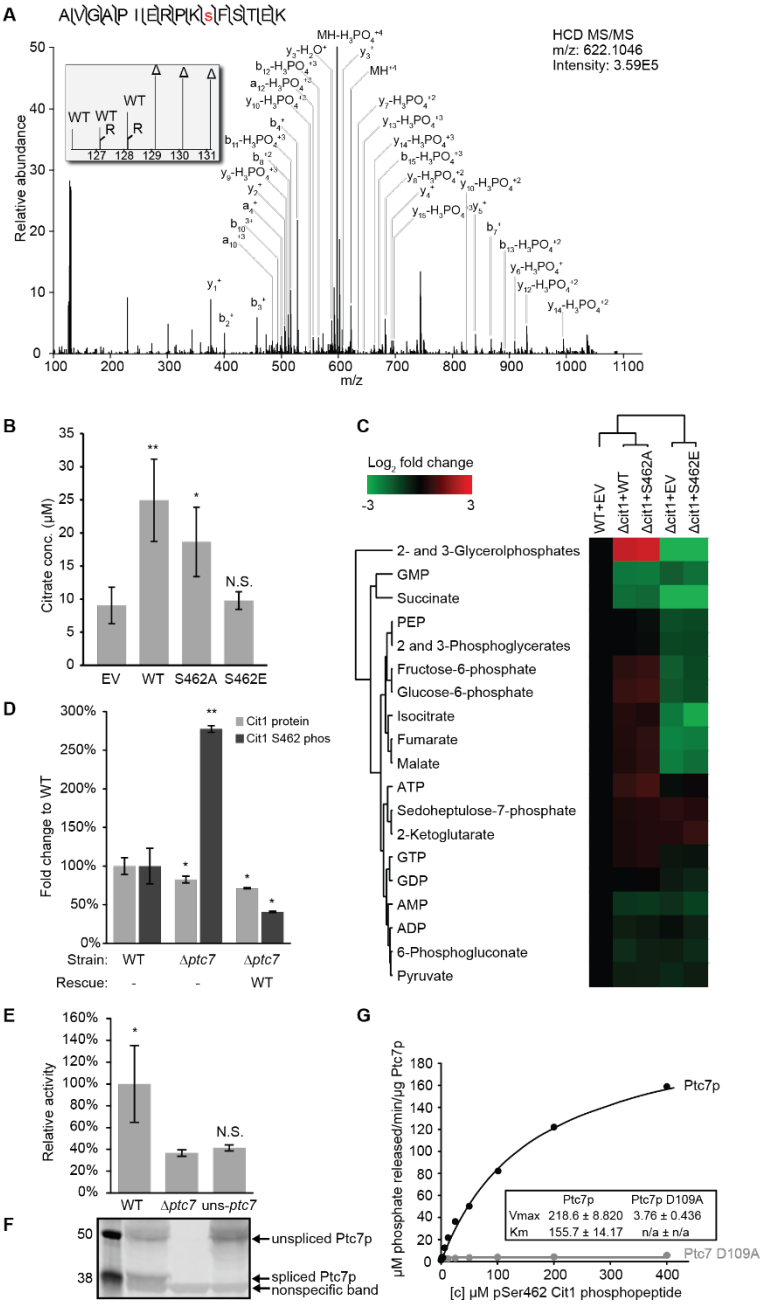

Figure S3, Related to Figure 3. A phosphomimetic mutant of Cit1p exhibits a distinguished metabolite profile.

(A) MS/MS spectrum identifies phosphorylation of serine 462 (S462) on Cit1p. The inset shows TMT reporter ions from eight channels representing wild type (WT), Δ*ptc7* (Δ) and Δ*ptc7* rescued with Ptc7p (R).
(B) Citrate concentrate of Δ*cit1* cell lysate expressing empty vector (EV), wild type (WT), S462A, or S462E Cit1p (mean ± SD, n=4).
(C) Hierarchical clustering of WT strain expressing empty vector, Δ*cit1* strain expressing EV, WT, S462A, and S462E Cit1p. Values are $\log_2$ fold change of corresponding strain compared to WT strain expressing empty vector (mean ± SD, n=3). GMP, guanosine monophosphate; PEP, phosphoenolpyruvate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; AMP, adenosine monophosphate; ADP, adenosine diphosphate.

(D) Fold change of both Cit1 protein and Cit1 S462 phosphorylation from MS dataset (mean ± SD, n=3 or 2). Rescue indicates if the strain is expressing a plasmid containing Ptc7p (WT: wild type Ptc7p).
(E) Citrate synthase activity of cell lysate from WT, Δ*ptc7*, or unspliceable *ptc7* (uns-*ptc7*) strains (mean ± SD, n=4). The p-value are calculated with comparison to the activity from Δ*ptc7* strain.
(F) Immunoblotting against HA (Ptc7p-HA) of cell lysate from WT, Δ*ptc7*, or uns-*ptc7* strains. Both WT and uns-*ptc7* have an endogenous HA tag on the c terminus of *ptc7*. The size of the protein ladder (molecule weight in unit of kDa) are labeled on the left side. Both spliced and unspliced Ptc7p are labeled on the right side. There is a nonspecific band right below spliced Ptc7p.
(G) Kinetic curve of *in vitro* phosphatase assay of recombinant Ptc7p or Ptc7p D109A on synthetic Cit1p phosphopeptide. The inset shows V_{max} and K_m values.
* p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; N.S., not significant.

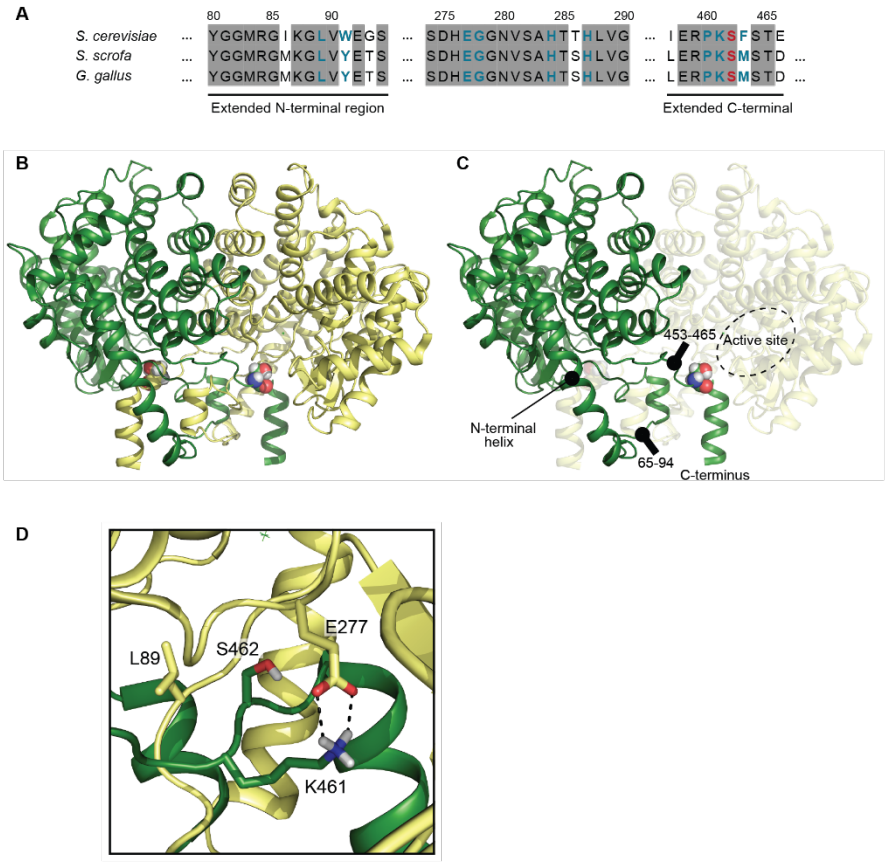

Figure S4, Related to Figure 4. Ser 462 belongs to an extended region of the dimer interface.

(A) The amino acid positions (highlighted in blue) that contact S462 (highlighted in red) are highly conserved among the yeast, pig and chicken proteins.
(B) Structure of dimeric citrate synthase. S462 is shown in spheres. Figure based on the structure of pig heart synthase (pdb code: 3ENJ).
(C) Same structure in which subunit A (green) has been enhanced, showing that part of the dimer interface consists of two extended and highly interdigitated regions of contact between the two monomers. These consist of the N-terminal helix (residues 46-64) followed by long unstructured region (65-94), and of a C-terminal unstructured region (453-465) followed by a C-terminal helix. Figure based on the structure of pig heart synthase (pdb code: 3ENJ), using yeast sequence numbering.
(D) Highlight of the region surrounding S462. The position is in proximity of an inter-subunit salt bridge (E277 and K461). S462 is in contact also with L89, in the extended N-terminal region of the opposite subunit.

SUPPLEMENTAL TABLE LEGENDS

Table S1, Related to Figure 2. Phosphoproteomics data.
Table of protein phosphoisoforms identified and quantified by LC-MS/MS and related information.

Table S2, Related to Figure 2. Proteomics data.
Table of proteins identified and quantified by LC-MS/MS and related information.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cloning of Ptc7 constructs and mutants

The full-length open reading form of *ptc7* (systematic name: YHR076w) was obtained from yeast genomic DNA and cloned into p416-GPD using EcoRI and HindIII restriction sites. To generate the spliced version of *ptc7*, 93 base pairs corresponding to amino acids 19-50 were eliminated using a PstI restriction-based method. The resulting ORF is 31 amino acids shorter than full length and corresponds to the *ptc7* spliced sequence as previously reported (Juneau et al., 2009). Catalytically inactive mutants were generated through the mutation of highly conserved aspartate residues required for metal binding in PP2C phosphatases (Bork et al., 1996). These two aspartates lie at positions D140/D109 (full length/spliced) and D228/D197 (full length/spliced). Each aspartate was mutated to an alanine using standard site directed mutagenesis (SDM) technique using the following primer sets: D109A fwd: GCCGGTGTCGCAGCTGGTGTTGGAGGATG, D109A rev: CATCCTCCAACACCAGCTGCGACACCGGC, D197A fwd: GCCAACTTGGGTGCTTCTTGGTGTGG, D197A rev: CCACACCAAGAAGCACCCAAGTTGGC. For recombinant protein expression, an N-terminal deletion of 38 amino acids (Nd38) was made to the *ptc7* ORF, corresponding to the probable mitochondrial targeting sequence (MTS) of Ptc7p as predicted by MitoProt II (Claros and Vincens, 1996). Nd38-*ptc7* was cloned into the pVP68K vector as previously described (Blommel et al., 2009) creating a fusion protein of the *ptc7* ORF with 8xHis and MBP tags followed by a tobacco etch virus (TEV) cleavage site (8His-MBP-TEV-Nd38-*ptc7*). Catalytically inactive mutants of Nd38-Ptc7p in pVP68K were generated using SDM and the primer sets for D109A and D197A shown above. All cloning products and mutants were verified by Sanger sequencing.

Ptc7p recombinant protein generation

pVP68K plasmids encoding wild type, D109A, and D197A *ptc7* were transformed into the BL21-CodonPlus(DE3) RIPL *E. coli* strain (Agilent Technologies) and selected on 15 µg/mL chloramphenicol (cam) and 50 µg/mL kanamycin (kan). Single colonies were picked into 6 mL starter cultures (LB media supplemented with cam/kan) and grown overnight at 37°C; these starter cultures were then diluted into 500 mL TB+G autoinduction medium and allowed to grow at 37°C for 2 hours as previously described (Fox and Blommel, 2009). Bacteria were then shifted to 25°C and grown overnight (~18 hours). Cells were collected at 6000 x g and either flash frozen or immediately lysed for protein prep. Recombinant proteins were purified as previously described, with minor changes (Stefely et al., 2015). Briefly, cells were resuspended in 25 mL lysis buffer (50 mM HEPES (pH 7.2), 150 mM NaCl, 5% glycerol, 5 mM BME, 0.25 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.5) and sonicated (30% amplitude, on ice; 20 s on, 40 s rest x 4 cycles per prep). Lysates were clarified by centrifugation (15,000 x g, 30 min, 4ºC). Talon resin was equilibrated in ES buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 5% glycerol, 5 mM BME, 0.25 mM PMSF, pH 7.2). Lysates were added to equilibrated buffer (1 mL resin/0.5 L culture) and incubated on a nutator at 4ºC for 1 hour. Protein-bound resin was washed 3x with ES buffer, followed by 3x washes in wash buffer (50 mM HEPES (pH 7.2), 150 mM NaCl, 5% glycerol, 5 mM BME, 0.25 mM PMSF, 10 mM imidazole, pH 7.2). His-tagged protein was eluted by incubating Talon resin in Elution buffer (50 mM HEPES (pH 7.2), 150 mM NaCl, 5% glycerol, 5 mM BME, 0.25 mM PMSF, 100 mM imidazole, pH 7.2) for 10 min at room temperature on a nutator. Beads were spun (1,000 x g) and supernatant was transferred to a 30 kDa MW spin-column (Amicon) to concentrate protein. A TEV cleavage reaction was performed overnight at 4ºC with constant rotation. His-MBP was subsequently purified from the reaction through reverse IMAC (Talon resin added and incubated with post-TEV reaction for 1 hour at 4ºC). Supernatant of this reaction was again concentrated using 30 kDa MW spin-columns, aliquoted, flash frozen in liquid nitrogen, and stored at -80ºC. Final elutions were quantified via Nanodrop (A280) and quantification verified through equal loading of ~100 ng protein on a 10% SDS-PAGE gel, followed by Coomassie staining.

Ptc7p-based phosphatase assays

Nd38-Ptc7p recombinant proteins and catalytically inactive mutants (D109A and D197A) were assayed for activity using the generic phosphatase substrate para-nitrophenyl phosphate (pNPP) (New England Biolabs). For pNPP-based phosphatase assays, 100 ng of phosphatase was incubated with 10 mM pNPP in the presence of 5 mM divalent cation (either $MgCl_2$ or $MnCl_2$) diluted in a final volume of 100 µl of 50 mM Tris (pH = 8.0). Addition of pNPP was used to initiate the reaction, and reactions lacking enzyme or divalent cations were set up as negative controls. The dephosphorylation of pNPP to para-nitrophenol (pNP) produces a colorimetric reaction which was followed by monitoring absorbance at 405 nm on a Cytation 3 plate reader (BioTek). A standard curve of pNP (Sigma-Aldrich) was created for normalization of A405 to µM pNP released. To determine biochemical parameters, various concentrations of pNPP were assayed with limiting amounts of Nd38-Ptc7p or mutants to generate linear curves. The

slopes of these curves (minimum 5 points) were calculated and the substrate concentration/catalysis rate pairs were used to generate Michaelis-Menten curves using the "ligand binding – one site saturation" fit model on SigmaPlot 13 software. For phosphopeptide-based phosphatase assays, an 11 amino acid residue containing phosphopeptide corresponding to Cit1 pS462 (amino acid sequence: IERPKpSerFSTEK) was obtained from Biomatik at greater than 90% purity. Phosphopeptide was reconstituted in water at a concentration of 1 mM, aliquoted, and stored at -80ºC until use. For the dephosphorylation reaction, 25 ng of Nd38-Ptc7p (wild type or D109A) was incubated with phosphopeptide (concentrations ranging from 0.4 to 400 µM), 5 mM $MnCl_2$, and 50 mM Tris (pH = 8.0). Reactions were allowed to proceed for 15 min at room temperature before determining free phosphate release through malachite green assays (Geladopoulos et al., 1991). Briefly, malachite green reagent (prepared as described in (Baykov et al., 1988)) was added at a volume of 20 µl to 80 µl phosphatase reaction (100 µl final volume). Malachite green absorbance was read in a 96 well plate at 621 nm on the Cytation 3 plate reader (BioTek). A standard curve of KH_2PO_4 was made to normalize A621 to µM free phosphate released. Kinetics were determined using the SigmaPlot software as described above.

Yeast strains used in this study

Haploid strains BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) were obtained from Open Biosystems and were used throughout this study. For our phosphoprotoemic analysis, we generated acute knockout and rescue strains for analysis from the parental yeast strain BY4741. The plasmids p416-GPD (vector only), *ptc7* in p416-GPD (wild type), and *ptc7* D109A in p416-GPD (catalytically inactive) were transformed into wild type BY4741 yeast as previously described (Gietz and Woods, 2002). Each strain was made null for *ptc7* through homologous recombination-based replacement of the *ptc7* ORF with a His3MX6 cassette (Longtine et al., 1998). For all experiments, Δ*ptc7* strains were freshly generated or streaked, grown on plates for 48 hours at 30°C, stored at 4°C and used within 1 week to prevent compensatory signaling processes. The Δ*cit1* strain used in this study was generated in the Stanford Genome Project deletion library (Winzeler et al., 1999) and was obtained from the BY4742 library commercially available through Open Biosystems. Wild type BY4742 yeast were used as controls for these experiments. To generate rescue strains, the *cit1* ORF including ~500 base pairs of its endogenous promoter was cloned from yeast genomic DNA. This fragment was ligated into a p416-based vector containing a C-terminal 6xHis/3xHA (a generous gift from Jared Rutter) using the restriction sites SacI and SpeI. The vector was modified to replace the C-terminal 6xHis/3xHA tag with a C-terminal FLAG tag through PIPE cloning (Klock and Lesley, 2009). S462 mutants (alanine or glutamate) were generated using SDM using the following primers: S462A fwd: AAAGGCCAAAAGCGTTCTCCACCGA, S462A rev: TCGGTGGAGAACGCTTTTGGCCTTT, S462E fwd: AAAGGCCAAAAGAGTTCTCCACCGA, S462E rev: TCGGTGGAGAACTCTTTTGGCCTTT. Empty vector, wild type *cit1*, S462A or S462E were all transformed into wild type BY4742 or Δ*cit1* BY4742 as previously described (Gietz and Woods, 2002). The marker-free unspliceable *ptc7* strain was made with the mutations and the methodology described previous (Juneau et al., 2009; Storici et al., 2001). In brief, the CORE cassette (KIURA3-KanMX4) was integrated into the genome to replace the intron of *ptc7*. A double stranded DNA containing the desired mutations in the intron region, was homologously recombined to replace the CORE cassette. The mutations were verified by sequencing. An endogenous three tandem hemagglutinin (3xHA) tag was introduced into both WT and unspliceable *ptc7* strains on the carboxyl terminus of Ptc7p by using pFA6a-3HA-His3MX6 plasmid and the method described before (Longtine et al., 1998).

Yeast growth assays

For all growth assays, yeast were transformed or freshly streaked onto uracil dropout (Ura^-) agar plates containing 2% dextrose (D, w/v). Individual colonies were selected into Ura^- 2%D liquid media (~3-4 mL) and incubated overnight (~14-16 hours, 30°C, 230 RPM). Cells were quantified by measurement of OD600 using a Nanodrop after a 1:20 dilution into a disposable cuvette. For drop assays, cells were serially diluted and plated at 10^4, 10^3, 10^2, and 10^1 on Ura^- agar plates containing either 2%D, 3% glycerol (G, w/v), or 2% sodium acetate (w/v), and incubated at 30°C for 2 to 4 days. For growth curves, yeast were diluted to a final concentration of 5 x 10^6 cells/mL in either Ura^- 2%D, Ura^- 3%G or Ura^- 0.1%D+3%G. 100 µl (final [c] cells = 5 x 10^5 cells/well) were transferred to a round bottom 96 well plate and sealed with a Breathe-Easy gas permeable sealing membrane (Sigma-Aldrich). Plates were transferred to a Cytation 3 plate reader (BioTek) and incubated at a constant temperature of 30°C with constant linear shaking (1096 cpm). OD600 were taken every 10 min for 16-24 hours. To quantify maximum yeast growth, the MaxV function of at least 5 points of the linear region of growth (~4 hours for glucose-containing cultures, ~12 hours for glycerol containing cultures) was calculated using the Gen5 2.09 plate reader software. This corresponds to the maximal slope

of growth across these time points, and is reported in mOD/hr. At least 3 replicates were quantified, and statistical significance was calculated using a Student's t-test on the growth rates quantified from these replicates.

Oxygen consumption assays

Oxygen consumption assays for yeast were performed using a Seahorse analyzer as previously described (He et al., 2013). Briefly, cells were grown in overnight cultures containing Ura$^-$ 2%D as described above (see *Yeast growth assays*). Cells were normalized to 4 x 10^6 cells/mL in fresh Ura$^-$ 2%D media, and 100 µl of these cells (final [c] cells = 4 x 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 1000 x g to sediment yeast, which were then incubated at 30°C for 30 min before being placed in the Seahorse XF-96 Extracellular Flux Analyzer. Oxygen consumption rate (OCR) was measured with a 1 min mix step and 3 min measure step for 3 rounds, with the final round of OCR reported. At least 3 replicates were quantified, and statistical significance was calculated using a Student's t-test on the growth rates quantified from these replicates.

Yeast growth for phosphoproteomics study

Yeast strains were generated as acute knockout and rescue strains as detailed (*yeast strains used for this study*) above. Importantly, 48 hours after transformation, individual colonies representing true biological replicates (independently derived knockouts) were selected directly into 100 mL Ura$^-$ 0.1%D 3%G media. For this study, 3x wild type colonies (BY4741 + p416-GPD), 3x Δptc7 knockout colonies (BY4741 *ptc7*:His3MX + p416-GPD), and 2x rescue colonies (BY4741 *ptc7*:His3MX + full-length *ptc7* in p416-GPD) were cultured. Each strain was grown to an OD~1 at 30°C with constant shaking (230 RPM) for ~16-24 hours, depending on the genotype of the strain (see Figure S2A). 10 mL of sample was saved for analysis of CoQ levels (see *Coenzyme Q6 and HAB analysis*), 10 mL of sample was saved for genomic DNA isolation and subsequent genotyping, and the remaining 80 mL was saved for proteomic and phosphoproteomic analysis. All samples were spun down at 1000 x g and the pellet flash frozen in liquid nitrogen. Samples were stored at -80°C until processed for mass spectrometry analysis. All samples were confirmed as proper genotypes at both the *ptc7* genomic locus, as well as for plasmid expression (empty vector or *ptc7*-expressing).

Sample preparation for LC-MS/MS

Samples were thawed on ice and resuspended in lysis buffer (8 M urea, 100 mM Tris (pH = 8.0), 10 mM tris(2-carboxyethyl)phosphine (TCEP), and 40 mM chloroacetamide (CAA)), followed by addition of methanol to 90% as final volume percentage. The mix was vortexed for ~30 s, and protein precipitate were collected after centrifugation (12,000 x g for 5 min). The protein pellet was resuspended in lysis buffer, vortexed for 30 min, and further diluted to 1.5 M urea with 50 mM Tris (pH = 8.0). Trypsin was added according to ~1:50 mass ratio to protein, and incubated overnight at room temperature. The digestion was quenched by adding 10% trifluoroacetic acid (TFA) to bring the pH below 2.0. The peptides were desalted using Sep-Pak Vac 1cc tC18 cartridges (Waters). The C18 columns were equilibrated in sequential order with 3 mL acetonitrile (ACN), 1 mL 70% ACN, 1 mL 40% ACN, 1 mL 20% ACN, and 3 mL 0.1% TFA. Samples were loaded, and followed by 3 mL 0.1% TFA wash. Peptides were eluted off by adding 1 mL 40% ACN, and then 0.75 mL 70% ACN. The elutions were dried in a speed vac, and resuspended in 0.5 mL 0.2% formic acid (FA). Pierce™ quantitative colorimetric peptide assay (Thermo) was used to determine peptide concentration. A total amount of 0.5 mg peptide from each sample was aliquoted out, and followed by 8-plex TMT labeling. Each sample was resuspended in 100 µL of 200 mM triethyl ammonium bicarbonate (TEAB, pH = 8.0), and each tag was resuspended in 50 µL ACN. After mixing sample with tag, incubation was done at room temperature for 2 hours while shaking. 8 µL of 5% hydroxylamine was added to each sample, and incubated at room temperature for 15 min while shaking to quench the reaction. A test mixture was analyzed by LC-MS/MS to confirm larger than 95% labeling efficiency before combining all samples. The mixture was dried down and ready for phosphopeptide enrichment.

Phosphopeptide enrichment and high pH reverse phase fractionation

Immobilized metal affinity chromatography (IMAC) with magnetic agarose beads (Qiagen) was used to enrich for phosphopeptide (Phanstiel et al., 2011). Dried samples were resuspended in 1 mL 80% ACN/0.1% TFA. The magnetic beads were washed three times with water, incubated in 40 mM (pH = 8.0) ethylenediaminetetraacetic acid (EDTA) for 30 min while shaking. The beads were then washed three times with water again, followed with incubating in 100 mM $FeCl_3$ for 30 min while shaking, and 4 times wash with 80% ACN/0.1% TFA. Resuspended samples were added into beads and incubated for 30 min while shaking. Flow-through was saved for protein analysis. The beads were

washed 3 times with 80% ACN/0.1% TFA. 400 µL 50% ACN/0.7% NH_4OH was added and vortexed for 1 min to elute phosphopeptides out of beads. The elution procedure was done twice, and combined to dry. Both IMAC flow-through and elution were further fractionated by a Gemini C18 reversed phase column (4.6 mm x 250 mm; Phenomenex). Mobile phase A (20 mM ammonium formate, pH = 10) and mobile phase B (20 mM ammonium formate in 80% ACN, pH = 10) were used to make gradient. Surveyor LC quaternary pump (Thermo) was applied to generate gradient with flow rate 0.8 mL/min. Samples were resuspended in 500 µL mobile phase A and injected into column. Fractions were collected every minute, dried out, and further combined to make a total of 10 fractions for both phosphorylation and protein analysis.

LC-MS/MS

Samples were analyzed by reverse phase liquid chromatography on a Dionex UltiMate UPLC system (Thermo) coupled to a Thermo Orbitrap Fusion (Thermo) (Hebert et al., 2014; Richards et al., 2015). A 75-360 µm inner-outer diameter silica capillary was packed with 1.7 µm diameter Bridged Ethylene Hybrid C18 particles (Waters). Mobile phase A (0.1% formic acid, and 5% dimethyl sulfoxide (DMSO) in water), and mobile phase B (0.1% formic acid, and 5% DMSO in acetonitrile) were made for a 90 min gradient running at 60^{o}C. MS1 scan with 60 K resolution and 5 x 10^5 AGC target was performed from 300 to 1250 m/z, followed with data dependent MS2 scans in a cycle time of 3 s. The precursors were isolated in 1.2 Th window, followed by HCD fragmentation with normalized collision energy of 35. The MS2 scan was set at resolution of 60 K with 5 x 10^4 AGC target. The maximum injection time was set up at 100 ms for MS1 and 300 ms for MS2. 60 s of dynamic exclusion time were applied, and precursor ions with +2 to +8 charge states are included for MS2.

Database searching and protein/phosphoprotein analysis

Coon OMSSA Proteomics Software Suite (COMPASS) was applied to analyze MS raw files (Wenger et al., 2011). MS raw files were first converted into .dta text files using DTA Generator, and searched against a target-decoy data base of both canonical and isoforms of saccharomyces cerevisiae proteome (.fasta file of database was downloaded from UniProt). A precursor mass tolerance of 150 ppm, a product ion mass tolerance of 0.01 Da, and up to 3 missed cleavages with trypsin were applied to searching based on Open Mass Spectrometry Search Algorithm (OMSSA) (Geer et al., 2004). Fixed modifications included carbamidomethylation of cysteines, and 8-plex TMT labeling on N-terminus and lysine residues. Variable modifications included oxidation of methionines and 8-plex TMT on tyrosines. Additional variable modification including phosphorylation with neutral loss on serine and threonine residues, and intact phosphorylation on tyrosine residues were applied to phosphopeptide searching. FDR Optimizer was utilized to filter peptides to 1% false discovery rate (FDR) with maximum 25 ppm mass error. TagQuant was used to assign TMT reporter ion intensities, with isotope purity corrections and normalization to total intensities in each channel. Protein Hoarder grouped peptides grouped into parsimonious protein groups at 1% FDR at the unique protein group level, and total reporter ion intensities of all PSMs within one protein group was used for protein intensity (Nesvizhskii and Aebersold, 2005). Phosphorylation localization was further decided by Phosphinator with ambiguity score threshold set at 13. Total reporter ion intensities of all localized phosphopeptides represented the intensity of one phosphoisoform. The intensity of phosphoisoform was normalized to the corresponding protein intensity. A database of yeast mitochondrial proteome (http://mitominer.mrc-mbu.cam.ac.uk/release-3.0/begin.do) was utilized to classify proteins as mitochondrial or non-mitochondrial.

Coenzyme Q6 and HAB analysis

A frozen pellet of yeast (1 x 10^8 yeast cells) generated from identical samples used for phosphoproteomic analysis (see *Yeast growth for phosphoproteomic study*) was thawed on ice and mixed with 200 µL phosphate buffered saline and 100 µL glass beads (0.5 mm diameter), followed by 30 s vortexing to lyse. 10 µL of 10 µM Coenzyme Q10 (CoQ10) was added as an internal standard, and the lysate was vortexed for 30 s again. 500 µL hexanes/2-propanol (10:1, v/v) was added and vortexed (2 x 30 s). The samples were centrifuged at 3,000 g for 1 min at 4 °C, and 400 µL of the organic phase was transferred to a clean tube and dried under N_2 (g). The residue was reconstituted in ACN/IPA/H2O (65:30:5, v/v/v) (100 µL) by vortexing (30 s) and transferred to a glass vial for LC-MS analysis. LC-MS analysis of CoQ6 and HAB was done as previously described (Stefely et al., 2015).

Citrate quantification by GC/MS

1x10^7 yeast cells were inoculated in to 50 mL Ura$^-$ 0.1%D 3%G media. After 21 h, OD600 was measured to determine cell density (OD600 of 1 corresponds to 10^7 cells/mL). 2 x 10^8 yeast cells were rapidly isolated by vacuum filtration

onto 1 nylon filter membrane (0.45 μm pore size, Millipore). The cells were then washed by 1 mL of phosphate buffered saline, and immediately submerged into 1.5 mL pre-cooled extraction solvent (ACN/MeOH/H_2O = 2/2/1 (v/v/v)) in a 2 mL plastic tube. The tubes were stored at -80°C before analysis. Yeast extract (50 μL aliquot) and internal standard (10 μL d4-citrate, 80 μM) were aliquoted into glass vials and dried by vacuum centrifuge (1 hr). The dried extracts were resuspended in pyridine (25 μL) and vortexed. 25 μL of N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) was added, and the sample was vortexed and incubated (60 °C, 60 min). Samples were then analyzed using a GC/MS instrument comprising a Trace 1310 GC coupled to a Q Exactive Orbitrap mass spectrometer. The thermal gradient started at 150 °C for 21 min, and then was ramped by 100 °C/min to 320°C over 1.7 min and then held for 10 min. Analytes were injected onto a 30 m TraceGOLD TG-5SILMS column (Thermo Scientific) using a 1:10 split at a temperature of 275 °C and ionized using electron ionization (EI). The mass spectrometer was operated in SIM mode monitoring 273 m/z and 276 m/z using a resolution of 30,000 (m/Δm) relative to 200 m/z.

Citrate synthase activity assay

Yeast pellets were collected by centrifugation at 4000 rpm (3220 x g) for 5 min, washed with 0.5 mL H_2O, and transferred to 1.5 mL Eppendorf tube. The rest of the procedure was done either on ice or at 4 °C. 300 μL lysis buffer (100 mM Tris (pH = 7.4), 1% (v/v) Triton X-100, 1 mM EDTA, 1 mM benzamidine hydrochloride, 1 mM PMSF, 1 x Protease Inhibitor Cocktail (Roche), 1 x PhosSTOP (Roche)), and 200 μL glass beads were added to the cell pellet containing tube. Five rounds of 1 min beating and 30 s resting were done to lyse the cells. The clarified cell lysate was collected after centrifugation at 16,000 g for 10 min. Bicinchoninic acid (BCA) assay (Thermo) was done to quantify the protein concentration. Cell lysates were normalized to the same concentration. Cytation 3 plate reader (BioTek) and a 96 well plate were used to read the colorimetric citrate synthase assay. 40 μL of 500 mM Tris (pH = 7.4), 2 μL of 30 mM acetyl CoA, 2 μL of 10 mM 5,5'- dithio-bis(2-nitrobenzoic acid) (DTNB) , 96 μL H_2O, and 50 μL cell lysate containing 2.5 μg total protein, were added into each well (Srere, 1969). 10 μL of 10 mM oxaloacetic acid (OAA) were added per well, and mixed by pipetting up and down. Absorbance at 412 nm (A412) was measured every 50 s. The initial slope was calculated by using data from the first 12 min, and used as enzyme reaction rate. For recombinant protein citrate synthase assays, 100 ng protein was incubated in the same reaction mixture as described above, but the concentration of OAA was adjusted (range of 0.5 to 500 μM) to facilitate kinetic measurements, as assayed by linear curves generated with limiting enzyme. The slopes of these curves (minimum 5 points) were calculated and the substrate concentration/catalysis rate pairs were used to generate Michaelis-Menten curves using the "ligand binding – one site saturation" fit model on SigmaPlot 13 software.

Homolog sequence alignment

Protein sequences were downloaded from the Uniprot (http://www.uniprot.org/) or NCBI (http://www.ncbi.nlm.nih.gov/) website. MacVector was used to alignment multiple protein sequences. ClustalW alignment algorithm was used with default settings.

Blue-Native gel and immunoblotting

Yeast cells were collected, lysed, and quantified the same way as described (see *Citrate synthase activity assay*). The following protocol is adopted from NativePAGE Bis-Tris gel electrophoresis protocol (Life Technologies). Briefly, 15 μL cell lysate was mixed with 5 μL 4 x NativePAGE sample buffer, and 1 μL of 5% NativePAGE G-250 sample additive. 10 μL of each sample were loaded to 4-16% NativePAGE Bis-Tris gel. 1 x NativePAGE anode buffer and 1 x NativePAGE dark blue cathode buffer were added into anode, or cathode chamber, respectively. The gel was run at 150 V for 1 h, then the dark blue cathode buffer was replaced with 1x light blue cathode buffer. The voltage was increased to 250 V until the dye front reached the bottom of the gel.

A standard western blotting protocol was done for regular SDS-PAGE. For Blue NativePAGE, after the transfer, the membrane was incubated in 20 mL 8% acetic acid for 15 min before a water wash, air dry, and rewet with methanol. Antibodies against FLAG (M2, Sigma-Aldrich with 1:5,000 dilution), HA (Thermo, 26183 with 1:10,000 dilution), and actin (Abcam, ab8224 with 1:1,000 dilution) were each used and incubated overnight at 4°C. Secondary antibodies diluted to 1:10,000 were used, and the LiCor Odyssey CLx imaging system was used to visualize the protein band.

Recombinant Cit1p protein prep

For recombinant protein expression, an N-terminal deletion of 48 amino acids (Nd48) was made to the *cit1* ORF, corresponding to the previously determined MTS of Cit1p (Vogtle et al., 2009). Nd48-cit1 was cloned into the pGEX-

6P-1 using BamHI and XhoI restriction sites to create a GST-Prescission Protease-Nd48 Cit1p fusion protein. S462A and S462E mutants were generated using SDM using the same primers pairs as described above (see *Yeast strains used in this study*). All cloning and mutagenesis was confirmed using Sanger sequencing. pGEX-6P-1 plasmids encoding wild type, S462A, and S462E *cit1* were transformed into the BL21-CodonPlus(DE3) RIPL *E. coli* strain (Agilent Technologies) and selected on 15 µg/mL chloramphenicol (cam) and 100 µg/mL ampicillin (amp). Single colonies were picked into 50 mL starter cultures (LB media supplemented with cam/amp) and grown overnight at 37°C; these starter cultures were then diluted into 500 mL LB media supplemented with cam/amp and allowed to grow at 37°C until bacteria reached log phase (OD600 ~ 0.7). Bacteria were then induced with 1mM final [c] IPTG, shifted to 25°C, and grown overnight (~18 hours). Cells were collected at 6000 x g and either flash frozen or immediately lysed for protein prep. Recombinant proteins were purified as previously described, with minor changes (Fuhs et al., 2015). Briefly, cells were resuspended in 1 mL GST lysis buffer (PBS, pH 8.0, 1% Triton X-100, 5% glycerol, 1 mM DTT) per 50 mL total culture and sonicated (30% amplitude, on ice; 20 s on, 40 s rest x 4 cycles per prep). Lysates were clarified by centrifugation (15,000 x g, 30 min, 4ºC). Glutathione resin was washed with GST lysis buffer clarified lysates were added to equilibrated buffer (1.5 mL resin/0.5 L culture) and incubated on a nutator at 4ºC for 1.5 hour. Protein-bound resin was washed 3x with GST lysis buffer, followed by resuspension in Prescission Protease Buffer (20 mM Tris pH=7.0, 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA). 10U of Prescission Protease (GE Healthcare) was added to each reaction and incubated overnight at 4ºC with constant rotation. After this overnight cleavage reaction, glutathione resin was spun (1000 x g) and supernatant was transferred to a 30 kDa MW spin-column (Amicon) to concentrate protein. Concentrated protein was then aliquoted, flash frozen in liquid nitrogen, and stored at -80ºC. Final elutions were quantified via Nanodrop (A280) and quantification verified through equal loading of ~100 ng protein on a 10% SDS-PAGE gel, followed by Coomassie staining.

Phosphoserine-incorporated Cit1p protein prep

To generate recombinant Cit1p with phosphoserine specifically incorporated at S462, we used a phosphoserine-incorporation system previously described (Pirman et al., 2015) with reagents provided as a kind gift from the Rinehart laboratory. N48-Cit1p in pGEX-6P-1, as described above, was used as the backbone expression vector for phosphoserine-incorporated protein. To generate "wild type" (non-phosphoserine incorporated) protein in the phosphoserine-expression system, the wild type sequence was used. To generate phosphoserine incorporated Nd48-Cit1p phosphoS462, the S462 codon was mutated to a "TAG" using the following primer pairs: S462TAG fwd: CTCCAATCGAAAGGCCAAAATAGTTCTCCACCGAAAAATAC and S462TAG rev: GTATTTTTCGGTGGAGAACTATTTTGGCCTTTCGATTGGAG. Either wild type or S462TAG Nd48-Cit1p in pGEX-6P-1 were co-transformed into C321.ΔA bacteria (a gift from Jesse Rinehart; Addgene # 68306) with the SepOTSλ (a gift from Jesse Rinehart; Addgene # 68292) and selected on LB agar plates supplemented with amp and kan. Transformed C321.ΔA bacteria were grown at 30°C. Single colonies were selected into 50 mL starter cultures (LB media supplemented with amp/kan) and grown overnight at 30°C; these starter cultures were then diluted into 1L LB media supplemented with amp/kan, 0.08% glucose, and 1.25 mM O-phospho-serine (Sigma-Aldrich). These cultures were allowed to grow at 30°C until bacteria reached log phase (OD600 ~ 0.7), and then were induced with 1mM final [c] IPTG, shifted to 23°C, and grown overnight (~18 hours). Proteins were purified using the same techniques as above (see *Recombinant Cit1p protein prep*).

PhosTag gel

Phosphoserine incorporation was validated by running samples on Phos-Tag acrylamide gel (Wako Biosciences) according to manufacturer's instructions. Before loading onto the PhosTag gel, both wild type and phosphoserine incorporated Nd48-Cit1p were subjected to phosphatase treatment using wild type or catalytically inactive (D109A) Nd38-Ptc7p. 2 µl Nanodrop-normalized Cit1p was incubated with 50 mM $MnCl_2$ in 50 mM Tris (pH=8.0). No enzyme, Ptc7p, or Ptc7p D109A were added (100 ng/reaction) to start the phosphatase reaction. Reactions were incubated at room temperature for 15 min before quenching the reaction with 4x Laemmli buffer and boiling the samples at 95ºC for 5 min. Samples (12.5 µl total) were then run on a 10% acrylamide gel supplemented with 100 µM Phos-Tag acrylamide (Wako Biosciences) according to manufacturer's instructions. PhosphoS462-Cit1p was visualized through a significant gel shift that was diminished by treatment with wild type Ptc7p, but not catalytically inactive protein.

Molecular modeling

A homology-based model of dimeric yeast Cit1p in the open state was generated from the pig heart structure (PDB code 3ENJ) using SWISS-MODEL (Biasini et al., 2014). This homology model as well as the pig heart (3ENJ) and

chicken (5CSC) structures were then minimized using BFGS constrained optimization. Serine 462 (424 in 3ENJ and 5CSC) was replaced with alanine, glutamate, and phosphoserine (using the SP2 CHARMM patch), and side chains were optimized using conformers from the Energy-Based Conformer Library (Subramaniam and Senes, 2012) with multiple iterations of a greedy algorithm using the CHARMM 22 van der Waals and electrostatic functions (Brooks et al., 2009). A conformer library for dianionic phosphoserine was generated by using the serine conformers in the Energy-Based Rotamer Library as a base (Subramaniam and Senes, 2012), and by sampling the $\chi 3$ rotation (dihedral rotation about the $O\gamma$-P bond) in increments of 10 degrees. All calculations were performed with programs written with the Molecular Software Library (Kulp et al., 2012).

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