Knockout of the Hmt1p Arginine Methyltransferase in *Saccharomyces cerevisiae* Leads to the Dysregulation of Phosphate-associated Genes and Processes

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**In Brief**
Arginine methylation of proteins in the eukaryotic cell is predominantly catalyzed by one conserved enzyme; PRMT1 in mammals or Hmt1p in yeast. Knockout in mammals is embryonic lethal; however, Hmt1p in yeast is non-essential. The systems-level effects of *hmt1* knockout in yeast were investigated. Unexpected but significant dysregulation in phosphate homeostasis was seen upon *hmt1* knockout. Transcription factor-driven processes may explain these observations, or regulatory processes may link the sensing of S-adenosylmethionine to intracellular phosphate or polyphosphate.

**Highlights**
- Knockout of arginine methyltransferase Hmt1p in *S. cerevisiae* was investigated.
- RNA-seq and SILAC MS/MS found downregulation of phosphate-associated processes.
- Phosphate homeostasis and extracellular levels of acid phosphatases were perturbed.
- Pho4p was an *in vitro* Hmt1p substrate, but this was not confirmed *in vivo.*
Knockout of the Hmt1p Arginine Methyltransferase in *Saccharomyces cerevisiae* Leads to the Dysregulation of Phosphate-associated Genes and Processes*$$\text{‡}$

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Hmt1p is the predominant arginine methyltransferase in *Saccharomyces cerevisiae*. Its substrate proteins are involved in transcription, transcriptional regulation, nucleocytoplasmic transport and RNA splicing. Hmt1p-catalyzed methylation can also modulate protein-protein interactions. Hmt1p is conserved from unicellular eukaryotes through to mammals where its ortholog, PRMT1, is lethal upon knockout. In yeast, however, the effect of knockout on the transcriptome and proteome has not been described. Transcriptome analysis revealed downregulation of phosphate-responsive genes in *hmt1Δ*, including acid phosphatases *PHO5*, *PHO11*, and *PHO12*, phosphate transporters *PHO84* and *PHO89* and the vacuolar transporter chaperone *VTC3*. Analysis of the *hmt1Δ* proteome revealed decreased abundance of phosphate-associated proteins including phosphate transporter Pho84p, vacuolar alkaline phosphatase Pho8p, acid phosphatase Pho3p and subunits of the vacuolar transporter chaperone complex Vtc1p, Vtc3p and Vtc4p. Consistent with this, phosphate homeostasis was dysregulated in *hmt1Δ* cells, showing decreased extracellular phosphate levels and decreased total Pi in phosphate-depleted medium. In *vitro*, we showed that transcription factor Pho4p can be methylated at Arg-241, which could explain phosphate dysregulation in *hmt1Δ* if interplay exists with phosphorylation at Ser-242 or Ser-243, or if Arg-241 methylation affects the capacity of Pho4p to homodimerize or interact with Pho2p. However, the Arg-241 methylation site was not validated in *vivo* and the localization of a Pho4p-GFP fusion in *hmt1Δ* was not different from wild type. To our knowledge, this is the first study to reveal an association between Hmt1p and phosphate homeostasis and one which suggests a regulatory link between S-adenosyl methionine and intracellular phosphate. *Molecular & Cellular Proteomics* 17: 2462–2479, 2018. DOI: 10.1074/mcp.RA117.000214.

Hmt1p is the predominant arginine methyltransferase in yeast (1). It has an abundance of ~37,600 copies per cell and is responsible for 66% of arginine monomethylation and 89% of asymmetric dimethylation of intracellular proteins (2, 3). Its substrates, identified through a series of proteome-scale and targeted approaches (4–7), include both histone and non-histone proteins. In the case of histone proteins, Hmt1p asymmetrically dimethylates histone H4 on Arg-3 (H4R3me2a) and contributes to the histone code through gene silencing (8, 9). On non-histone proteins, Hmt1p-mediated mono- and asymmetric di-methylation of arginine is implicated in transcriptional regulation, nucleocytoplasmic shuttling of proteins and mRNA, and to a lesser extent RNA processing and translational regulation (reviewed in (10)). In total, Hmt1p has been shown to have at least 25 substrate proteins, 10 of which have been validated in *vitro* (10, 11).

Hmt1p is conserved across model eukaryotes (such as *Arabidopsis thaliana*, *Drosophila melanogaster*, *Caenorhabditis elegans*) (12). In mammals, the predominant mammalian arginine methyltransferase, PRMT1, is the ortholog of Hmt1p and is involved in functions including genome integrity, cell proliferation and response to DNA damage (13). Interestingly, the dysregulation of PRMT1 is associated with human diseases such as cardiovascular disease and cancer (14) and in mice, knockout of PRMT1 is embryonic lethal (15). Despite arginine methylation being implicated in a plethora of biological processes, *HMT1* in *S. cerevisiae* is not essential. The knockout has not been documented to show a clear metabolic phenotype (16), but has been reported to show increased transcription from silent chromatin regions because of loss of H4R3me2a (17) and higher tRNA abundance compared with the wild-type (18).

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Phosphate is a critical macronutrient that is required for energy generation via ATP and GTP synthesis and DNA, RNA and phospholipid biosynthesis. It is also important for cellular signaling processes via phosphor transfer reactions that affect protein function and activity. In yeast, the phosphate (PHO) pathway is modulated by phosphate availability in the environment and the phosphate requirements within the cell. The PHO pathway is regulated by the transcription factor Pho4p and its target genes involve those that encode for membrane-embedded phosphate transporters PHO84, PHO89, PHO87, PHO90, PHO91, acid phosphatases PHO5, PHO11 and PHO12, and polyphosphate synthetases and transporters VTC1, VTC2, VTC3, and VTC4 (19, 20). Under phosphate limitation Pho4p becomes activated and transcribes extracellular phosphatases and transporters that sequester extracellular inorganic phosphate (Pi) from phosphate containing organic compounds and imports this Pi into the cell (19). Within the cell, excess cytosolic Pi is usually synthesized into polymers of Pi known as polyphosphates (polyP)

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To discover novel functions of Hmt1p, here we first investigated the changes in the transcriptome and proteome in the hmt1Δ deletion mutant, which revealed an apparent dysregulation in phosphate homeostasis. In hmt1Δ, many components of the PHO pathway, including repressible acid phosphatases, phosphate transporters and vacuolar transporter chaperone proteins showed downregulation and/or lower abundance in hmt1Δ as compared with wild-type yeast during mid-log growth. These results were associated with a decrease in phosphate production, a decrease in total Pi, in phosphate depleted medium, and the dysregulation of polyP homeostasis within the hmt1Δ cell. We further showed that the transcription factor Pho4p can be methylated, at Arg-241, by in vitro incubation with Hmt1p. Our study is the first to establish an association between Hmt1p-mediated arginine methylation, the regulation of the PHO pathway and of phosphate metabolism.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions—**Saccharomyces cerevisiae BY4741 haploid strain (Open Biosystems, Huntsville, AL; MATA his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was used as the wild-type in all experiments in this study unless otherwise specified. HMT1 knockout yeast (Open Biosystems; hmt1:: KANMX4 in BY4741) was used for studies related to deletion of arginine methyltransferase Hmt1p unless otherwise specified. Cells were maintained, and selection performed according to previous methods (27).

Strains were grown in YEPD (2% (w/v) d-glucose, 2% (w/v) bacto-tryptone, 1% (w/v) yeast extract) at 30 °C with orbital shaking at 200 rpm.

For SILAC experiments, a BY4741 lys2Δ/arg4Δ strain was used where genes in the lysine and arginine biosynthetic pathways had been knocked out. This strain served as the background strain. HMT1 was deleted from the BY4741 lys2Δ/arg4Δ strain by use of a hygromycin B resistance cassette amplified from plasmid pFA6a-hphNT1 as described in (28).

**Gene Expression Analysis and Raw Reads Filtering**—Gene expression analysis was performed on three biological replicates each of wild-type and hmt1Δ, sampled at mid-logarithmic growth phase (O.D.660 = 0.8–1.0). Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. Ribosomal RNA depletion was performed prior to library generation (Ribominus Eukaryote kit, Life Technologies). The cDNA libraries for HiSeq 2000 sequencing were constructed from 10 µg of total RNA using the TruSeq SBS Kit v3-HS (Illumina, San Diego, CA) according to the manufacturer’s instructions, generating 101 bp paired-end reads from a 160 bp insert library. RNA-Seq sequencing was performed using a HiSeq 2000 (Illumina), in the Ramaciotti Centre for Genomics, the University of New South Wales. Initial quality assessment for Illumina HiSeq sequence data was based on FastQC (version 0.11.2) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Pair-end raw reads were trimmed with the BWA trimming mode at a threshold of Q13 (p = 0.05) as implemented by SolexaQA version.
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1.11 (30). Low quality 3'-ends of each read were filtered. Reads that were less than 25 bp in length were discarded.

Mapping of RNA-Seq Reads and Differential Expression Analysis—Filtered reads from six samples were aligned to the *S. cerevisiae* S288C reference genome (version R64–1–1) (31) with TopHat 2.0.4 (32) and Bowtie 2–2.0.0-beta7 (33) using default parameters. Count files of the aligned sequencing reads were generated by the HTSeq-count script from the Python package HTSeq (34) with intersection-nonempty mode, using the GFF annotation file downloaded from the Saccharomyces Genome Database (35). Differential gene expression analysis was performed on the count files using the DESeq package (36) from Bioconductor (37), following standard normalization procedures. Genes with less than ten read counts in each replicate in both wild-type and hmt1Δ were removed from further analysis. Only genes differentially expressed at a false discovery rate ≤ 0.1 and adjusted p value < 0.05 were considered as significantly differentially expressed genes in the analysis.

Microarray Analysis—Total RNA was extracted from four wild-type and three hmt1Δ biological replicates using the Qiagen (Hilden, Germany) RNeasy isolation kit following mechanical disruption with 0.5 mm glass beads. Microarray analysis was performed in the Rama-cott Centre for Genomics (University of New South Wales) using the Affymetrix GeneChip*®* Yeast Genome 2.0 Array. Microarray data were analyzed using R/Bioconductor (37), the '.CEL' data files imported using the affy package (v3.10.4) (39). Scripts from Gillespie et al. (39) were used to mask the *S. pombe* probes, to extract the data for the *S. cerevisiae* probes. The intensity values from the *S. cerevisiae* probes were normalized using the Robust Multi-array Average (RMA) (40) function from the affy package. The quality of the normalized data was assessed using arrayQualityMetrics (v3.30.0) (39). Scripts from Gillespie et al. (39) were used to compute the average normalized values from multiple probes of the same gene. The differential expression of genes between the hmt1Δ and wild-type control yeasts was analyzed using limma (v3.30.4) (41). The Remove Unwanted Variation (RUV4) tool (v0.9.6) (42) was used to remove unwanted variation from the microarray data, with 500 of the least differentially expressed genes identified from an initial limma analysis used as empirical negative control genes. Two unwanted factors identified using RUV4 were removed using the linear model and a subsequent limma analysis was used to identify a final set of differentially expressed genes. The p values were adjusted using the Benjamini-Hochberg procedure.

Protein Extraction, 1-D Gel Electrophoresis, and Immunoblotting—Cells were harvested, lysed, and proteins electrophoresed according to established methods (44). The peptide digest pool was vacuum-dried (Savant SPD1010, Thermofisher Scientific, Waltham, MA) before re-dissolving in 4 ml MM-KH2PO4 or MM-KCl media at OD500 = 1.0 and incubated at 30 °C for 2 h. Next, cells were pelleted and resuspended in 3.2 ml of pNPP solution (2.5 mM pNPP) and washed twice in sterile water. To induce a response to high or low phosphate, cells were then resuspended in 4 ml MM-KH2PO4 or MM-KCl media at OD500 = 1.0 and incubated at 30 °C for 2 h. Next, cells were pelleted and resuspended in 3.2 ml of pNPP solution (2.5 mM pNPP (Sigma-Aldrich, St. Louis, MO) in 50 mM NaOAc, pH 5.2) and incubated at 37 °C. Every 30 min until 180 min, a 400 μl cell and level 4 GO terms categories were examined, to find terms with a p value of < 0.05.

Experimental Design and Statistical Rationale—To determine the proteome-level consequences of the deletion of *HMT1*, four parallel cultures (two of each of the background strain and hmt1Δ in heavy and light media) were used in SILAC experiments, producing two biological replicates. After the normalization of protein ratios in MaxQuant (45), the resultant data set of ~1500 proteins with quantitative information was then subjected to filtering, prior to any statistical analysis. The following rules were applied:

a) If a protein was identified with three or more peptides, any peptides with ratios higher than two standard deviations away from the mean for that protein were considered outliers and excluded.

b) Proteins identified with less than two peptides were excluded.

c) Proteins identified in only one dataset (A or B) were excluded.

d) All contaminants (e.g. keratins, trypsin) were excluded.

Post-filtering, mean protein ratios were then recalculated for the remaining 1375 proteins, and re-normalized to 1.0 using the limma package in R/Bioconductor (37). Empirical Bayes-moderated p values for each protein were finally calculated in limma; this approach does not assume any fixed distribution of protein ratios.

For RNA-Seq, biological triplicates of wild-type and hmt1Δ yeast were grown, extracted and then analyzed by HiSeq 2000 next-generation sequencing. For microarray analysis, biological quadruplicates of wild-type and biological triplicates of hmt1Δ yeast were grown, RNA was extracted and then analyzed. Analysis of gene expression data was as detailed above.

Acid Phosphatase Assay—Extracellular acid phosphatase activity was quantified by the hydrolysis of *p*-nitrophenyl phosphate (pNPP) to *p*-nitrophenol as per Lev et al. (48) or Orkwis et al. (49), with modifications. Cells from overnight cultures in YEPD were pelleted and washed twice in sterile water. To induce a response to high or low phosphate, cells were then resuspended in 4 ml MM-KH2PO4 or MM-KCl media at OD500 = 1.0 and incubated at 30 °C for 2 h. Next, cells were pelleted and resuspended in 3.2 ml of pNPP solution (2.5 mM pNPP (Sigma-Aldrich, St. Louis, MO) in 50 mM NaOAc, pH 5.2) and incubated at 37 °C. Every 30 min until 180 min, a 400 μl cell and peptide and protein false discovery rate of 0.01. No fixed modifications were used. For search tolerances, MS1 was set to < 5 ppm and MS2 was set to 0.40 Da. Functional Analysis of Differentially Expressed Genes and Proteins—Gene ontology (GO) terms for all differentially abundant proteins or differentially expressed genes, from proteomics and RNA-Seq experiments respectively, were extracted and overrepresented functional categories were determined by use of GOMiner (47). All unique identified proteins and genes served as the background list, and level 4 GO terms categories were examined, to find terms with a p value of < 0.05.

Expression of proteins was induced overnight and 300 ml of culture was pelleted, resuspended in binding buffer (50 mM Tris-HCl buffer pH 8.0, 50 mM NaCl, 40 mM imidazole, 20% (v/v) glycerol, 0.25% (v/v) Triton X-100, supplemented with EDTA-free protease inhibitor (Roche, Basel, Switzerland)), lysed and clarified as described previously (43). Ppx1p was His-purified via 1 ml Ni NTA cartridges (Qiagen) precharged with Ni2+-Sepharose for affinity purification ac-
cording to manufacturer’s instructions. The final eluate was concen-
trated using the Amicon Ultra-4 10K centrifugal filter (Merck Millipore,
Burlington, MA) at 25 °C and buffer exchanged with 50 mM Tris–HCl
(pH 7.4), 200 mM NaCl to reduce NaCl to at least 200 mM and remove
imidazole. Glycerol was added to a final concentration of 50% (v/v)
before storage at −80 °C.

To monitor the cellular concentration of polyphosphates (polyP) in
the wild-type and hmt1Δ mutant during growth, overnight cultures of
cells were subcultured into fresh YEPD at O.D.600 = 0.2 and MM-KCl
and MM-KH2PO4 at O.D.600 = 1.0. polyP was extracted at the lag (3 h
from subculture), log (7 h) and stationary phases (10 h) of growth in
YEPD as well as at 25 h after subculture. polyP in cells grown in
MM-KCl and MM-KH2PO4 were extracted at the same harvesting
times as for YEPD. Equal numbers of cells were adjusted by cell
density for polyP extraction and purification and quantification were
performed as described previously by Canadell et al. (50) with previ-
ously purified ScPpx1p.

To quantify total phosphate levels, cells were grown to O.D.600 = 1.0 in YEPD. Total phosphate levels were also quantified from MM-
KCl and MM-KH2PO4 after 3 h of subculture from YEPD at a starting
O.D.600 = 1.0. Equivalent numbers of cells were washed with sterile
Milli Q water, boiled in 1M H2SO4 for 20 min and assessed using P;
ColorLock™ Gold (Innova Biosciences, Cambridge, UK) according to
manufacturer’s instructions. Both polyphosphate and total phosphate
quantification assays were performed with two technical replicates in
at least three biological replicates.

Expression and His-purification of Pho2p and Pho4p—PHO2 and
PHO4 were cloned from BG1805 plasmids (Thermo Fisher Scientific)
to pRS725MCS1 (51) (see supplemental Table S2 for plasmids)
using PhoLinkF and PhoR primers specific for PHO2 and PHO4
respectively (see supplemental Table S3 for primers). The vector
pRS725MCS1 was amplified using DuetPstF and DuetHisRlink prim-
ers. PHO2 and PHO4 were cloned separately into the vector using
Gibson assembly cloning kit (New England Biolabs, Ipswich, MA),
according to manufacturer’s instruction. Assembled plasmids were
transformed into Alpha-select Gold efficiency competent cells (Bio-
line, London, UK). Transformants were plated onto LB plates with 50
µg/ml kanamycin for selection. Successful assembly was screened
with PCR using T7 promoter and DuetDOWN1 primers (supplemental
Table S3) and confirmed with Sanger sequencing.

Plasmids were grown, extracted and then transformed into BL21
Rosetta (DE3) E. coli cells for protein expression. Plasmids carrying
HMT1 and NPL3; a known substrate of Hmt1p (52), were also trans-
formed into the BL21 Rosetta (DE3) expression cells. All proteins were
overexpressed by growing in LB at 37 °C with orbital shaking at 200
rpm and inducing expression of proteins by 1 mM IPTG. Protein
induction was left for 6 h after which cells were pelleted and then
lysed by cell sonication in binding buffer (50 mM Na-phosphate buffer
pH 8.0, 0.5 mM NaCl, 40 mM imidazole, 20% (v/v) glycerol, 0.25% (v/v)
Triton X-100, 10 mM β-mercaptoethanol), supplemented with EDTA-
free protease inhibitor (Roche). The lysates were clarified by cen-
fugation (16,000 g, 20 min, 4 °C) and put onto HisTrap HP 1 ml
columns (GE Healthcare, Chicago, IL). The final eluate was concen-
trated using centricron Plus-20 centrifugal column (Merck Millipore)
at 4 °C, subjected to buffer exchange with 50 mM sodium-phosphate
buffer pH 7.4, 0.2 mM NaCl, 20% (v/v) glycerol and stored as detailed
previously.

Immunoprecipitation of Chromosomally GFP Tagged Pho4p—The
Pho4p-GFP strain was cultured in MM-KCl or MM-KH2PO4 to induce
a high and low phosphate response, as above, before the Pho4p-GFP
fusion protein was immunoprecipitated using GFP-Trap®_MA
(ChromoTek, Munich, Germany). Cells were lysed in lysis buffer (10
mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA 0.5% Triton X-100,
2.5 mM MgCl2, 20 U/ml DNase (New England Biolabs), cOmpleteTM
mini EDTA-free protease inhibitor (Roche), and PhoSTOP™ phospha-
tase inhibitor (Roche)) by bead-beating 3 times for 30 s with 3 min
incubations on ice between steps. Lysates were then clarified
(21,000 × g for 40 min at 4 °C) and filtered through a 0.45 µm filter.
25 µl of GFP-Trap®_MA bead slurry was washed three times with 500
µl chilled wash buffer (10 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 mM
EDTA) before 2.5 mg of lystate was added to beads and incubated for
1 h at 4 °C with end-over-end mixing. Beads were washed three more
times before boiling in 50 µl 2X SDS-PAGE sample buffer for 10 min.
Eluates were separated by 1-D electrophoresis and the band corre-
spanding to Pho4p-GFP was analyzed by mass spectrometry as
described below.

In Vitro and In Vivo Methylation and ETD-MS/MS Analysis—All
assays were performed essentially as described in (27), using recom-
binantly generated Hmt1p (53). Methylation reactions were incubated
at 30 °C for 2 h. Negative controls for each reaction were performed
with the omission of SAM (S-adenosyl methionine) in a replicate
reaction, substituted with water. Electron transfer dissociation (ETD)
analysis of in vitro protein methylation was conducted on an LTQ
Orbitrap Velos Pro ETD (Thermo Fisher Scientific) as described pre-
viously (53). MS-Digest (version 5.19.1, University of California, San
Francisco) outputs were used to calculate theoretical m/z values
associated with doubly and triply charged arginine methylated pep-
tides; these m/z values were incorporated into LC-MS/MS inclusion
lists for use in mixed targeted and data dependent acquisition LC-
MS/MS experiments. Theoretical Pho2p or Pho4p peptide masses
were generated in MS-Digest using the following parameters: trypsin
digest (up to two missed cleavages); variable modifications of carb-
/amidomethyl (C), Methyl (R), Dimethyl (Uncleaved R), Oxidation (M),
Phospho (STY); peptide masses 700 to 8000; and a minimum peptide
length of 5. No fixed modifications were used.

For analysis of in vitro protein methylation data, files were submit-
ted to the database search program Mascot (version 2.3, Matrix
Science). Searches and verification of methylpeptides by manual
examination for neutral losses were performed according to (5); in-
strument type was set as ETD-TRAP; precursor and MS/MS toler-
ances were ±4 ppm and ±0.5 Da, respectively; variable modifications
of acrylamide (C), carbamidomethyl (C), Oxidation (M), Methylation
(R) and demethylation (R) was used; digestion with trypsin was spec-
ified with two or four allowed missed cleavages; and the SwissProt
data-base was searched. No fixed modifications were used. For the vali-
dation of in vitro methylated peptide of Pho4p, a peptide of sequence
RmetSSGALVDDDKR was chemically synthesized (ChinaPeptides,
Shanghai, China) and analyzed as above. Analysis of in vivo methyla-
tion of Pho4p involved the overexpressing the His-tagged protein on
a BG1805 plasmid in wild-type BY4741 yeast. This and the affinity
purification of Pho4p was done according to (54). The resulting pro-
tein, as well as Pho4p-GFP immunoprecipitated as described above,
were prepared for mass spectrometric analysis as described previ-
ously (53). Peptide samples were analyzed using an UltiMate 3000
HPLC and autosampler system (Dionex) coupled to a Fusion Lumos
Tribrid (Thermo Fisher Scientific). Nano-LC and nano-ESI were per-
formed following experimental procedures described previously (53).
Survey scans m/z 350–1750 were acquired in the Orbitrap (resolu-
tion = 120,000 FWHM) with an AGC target value of 4 × 106 charges
(maximum ion injection time = 50 ms). Peptide ions (>5000 counts)
with charge states of 2–8 were sequentially isolated and fragmented
via ETD performed with a 100 ms reaction time, supplemental acti-
vation employed (ETciD at 10% collision energy) and a fluoranthene
anion target of 6 × 105. Fragment ions were mass analyzed in the
linear ion trap. Dynamic exclusion was applied to ions subjected to
MS/MS using the following parameters: exclude after n = 1, exclusion
duration = 25 s and mass tolerance = 10 ppm. A mixed targeted and
Site-directed Mutagenesis of PHO4 and HMT1—The Arg-241 (R241) of Pho4p was mutated to lysine in pRSF25 MCS1-Pho4 (supplemental Table S2). This was performed by use of Site-directed Ligase Independent Mutagenesis (SLIM) developed by Chiu et al. (55) with primers listed in supplemental Table S3. Plasmids were mixed and subjected to DpnI restriction enzyme digestion (New England Biolabs) to eliminate template DNA. Ten microliters from each PCR reaction were combined and hybridized. A hybridization program consisting of a denaturing step at 99 °C for 5 min, 3 cycles at 65 °C for 5 min and 30 °C for 45 min was used. The hybridization products were transformed into Alpha-select Gold efficiency competent cells (Bioline) at 5 μl of hybridization product per 50 μl of competent cells. Verification of the SLIM-modified plasmids was performed via colony PCR screening followed by Sanger sequencing. Next, mutated PHO4 gene was amplified from pRSF25 MCS1 using Pho4_Fwd145 and Pho4_Rev173 primers, whereas G68R_HMT1 was amplified from DuetHmt1G68R-Npl3T18 (51) using Hmt1_Fwd145 and Hmt1_Rev173 primers (supplemental Table S3). Separately, the vector pRS426, containing URA3 marker, was amplified using p426_Fwd173 and p426_Rev145 primers. The fragments were assembled using Gibson assembly cloning kit (New England Biolabs), subjected to DpnI digestion, plasmid hybridization as above, and then screened for successful incorporation of mutated PHO4 or HMT1 into pRS426 using p426_Ura primers. Subsequently, mutated PHO4 or HMT1 was amplified alongside URA3, using Fwd_Pho4/Rev_URA3 or Fwd_Hmt1/Rev_URA3_Hmt1 primers, respectively, containing flanking regions upstream and downstream of transformation target site as described in (28). The PCR products were transformed into wild-type S. cerevisiae as described in (56) and plated onto SC-URA plates (0.192% (w/v) yeast synthetic drop-out without uracil (Sigma-Aldrich), 0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulfate (BD Biosciences), 0.5% (w/v) ammonium sulfate, 2% (w/v) d-glucose, 2% (w/v) agar). DNA was extracted from successful transformants, screened with Pho4_A/Pho4_D or Hmt1_Check_A/Hmt1_Check_D primers, and finally confirmed with Sanger sequencing.

Fluorescence Microscopy—Low phosphate YEPD (1% (w/v) yeast extract, 2% (w/v) bacteriological peptone, 2% (w/v) d-glucose and 0.246% (w/v) MgSO4, pH 6.5) was prepared by chelating phosphates with 8 ml of concentrated NH4OH. After 30 min at room temperature, the solution was filtered twice through Whatman paper before filter sterilization. Wild-type, Tef1p-GFP, Pho2p-GFP, Pho4p-GFP and hmt1Δ Pho2p-GFP and hmt1Δ Pho4p-GFP cells were grown overnight in YEPD, washed twice with water and resuspended in a 1:10 dilution of low phosphate YEPD with and without 10 mM KH2PO4. The cultures were incubated for 5–6 h at 30 °C with shaking. The localization of Pho4p was viewed in the specified growth media at room temperature under an inverted fluorescence microscope (Olympus IX71), fitted with a CoolSNAP HQ2/ICX285 camera (Photometrics, Tucson, AZ). For each strain, 20 images of representative cells were taken to quantify the nucleolar localization of Pho4p. Pho2p-GFP and Tef1p-GFP, which are proteins localized in the nucleus and cytoplasm, respectively, were included in fluorescence microscopy for comparison. Nuclei were stained by the addition of 1 μg/ml DAPI in the media for 20–30 min prior to viewing. Statistical analysis of Pho4p-GFP nucleolar localization was performed using the student’s unpaired t test, where p < 0.05 was considered significant.

Images were taken under an Olympus 100x OIL/1.40 objective. Image acquisition and deconvolution were performed with the Resolve3D softWoRx-Acquire (version 6.5.2) imaging software. Images were taken after 1 s of exposure, with a FITC filter for GFP fluorescence (475/28 nm excitation and 523/36 nm emission) and DAPI filter for nuclear fluorescence staining (390/18 nm excitation and 435/48 nm emission).

RESULTS

Transcriptome Analysis of hmt1Δ Yeast Reveals Downregulation of Genes Associated with Phosphate Homeostasis—To investigate the effects of Hmt1 gene deletion on the transcriptome, we first performed RNA-Seq analysis. Global gene expression in wild-type and hmt1Δ yeast at O.D.600 = 0.8–1.0 was directly compared, with an average of 21.9 and 24.8 million reads generated for the biological triplicates of wild-type and hmt1Δ, respectively. Filtered RNA-Seq reads were aligned to the yeast genome and differential gene expression analysis performed using DESeq (36). A total of only 17 genes were found to be significantly differentially expressed between wild-type and hmt1Δ (Table I). Four genes showed up-regulation, whereas 13 genes including HMT1 showed downregulation in hmt1Δ. HMT1 showed more than a 1000-fold difference in read counts between wild-type and hmt1Δ yeast, confirming its knockout.

We investigated any functional relationships between differentially expressed genes using GOMiner (47). Six of the 17 genes were of unknown function. Analysis of the 11 remaining genes revealed that acid phosphatase activity and phosphate metabolic process were significantly downregulated (Fig. 1A and supplemental Table S4 for genes in each category). Genes involved were the acid phosphatases PHO5, PHO11, PHO12, the phosphate permease PHO89 and cyclin-dependent kinase inhibitor SPL2. Interestingly, all are PHO (phosphate-responsive signaling) regulated and known to be controlled by transcription factor Pho4p (19). The VTC3 member of the vacuolar transporter chaperone, involved in intracellular polyphosphate generation (19, 59, 60), was also significantly downregulated.

Validation of the hmt1Δ RNA-Seq data via microarray confirmed seven significantly downregulated genes that were also differentially expressed in RNA-Seq. These were HMT1, FDC1, IMD2, SIZ1, SPL2, PHO11, and PHO5 (Table I). Additionally, other phosphate-associated genes were downregulated although their fold changes were not significant. These included PHM6, PHM8, PHO3, PHO8, PHO84, PHO86, PHO89–91, VTC1–5, and IZH2 (61) (supplemental Fig. S1). Taken together, RNA-Seq and microarray analysis revealed a downregulation of the PHO pathway in hmt1Δ, raising the prospect that Hmt1p-mediated methylation affects phosphate homeostasis in the cell.

Deletion of HMT1 Leads to Downregulation of Proteins Associated with Phosphate Homeostasis—To determine if the gene expression changes in hmt1Δ affected the proteome, and in particular the PHO pathway, we used SILAC MS/MS. S. cerevisiae strain BY4741 lys2Δ/arg4Δ was used as background with critical steps in lysine and arginine biosynthesis knocked out to allow complete isotopic labeling. We then deleted the gene for methyltransferase Hmt1p in that back-
| Gene ID   | Description                        | RNA-Seq analysis | Microarray analysis |
|----------|-------------------------------------|------------------|---------------------|
|          |                                     | Normalised count | Normalised count    |
|          |                                     | wild-type\(^a\)  | hmt1\(^\Delta\)    |
|          |                                     | Fold Change (FC) | Log\(_2\) (FC)     |
|          |                                     |                  | \(p_{\text{adj}}\) |
| Upregulated in hmt1\(^\Delta\) |                      |                  |                     |
| CO512    | Uncharacterized protein              | 15.8             | 117.3               |
|          |                                     | 7.42             | 2.89                |
|          |                                     | 1.07E-16         |                     |
|          |                                     |                  |                     |
| YFL067W  | Uncharacterized protein              | 16.4             | 48.1                |
|          |                                     | 2.93             | 1.55                |
|          |                                     | 1.73E-02         |                     |
|          |                                     |                  |                     |
| YHR214W-A| Putative uncharacterized protein     | 62.2             | 151.5               |
|          |                                     | 2.43             | 1.28                |
|          |                                     | 3.44E-04         |                     |
|          |                                     |                  |                     |
| SIZ1     | E3 SUMO-protein ligase               | 221.2            | 532.8               |
|          |                                     | 2.41             | 1.27                |
|          |                                     | 5.84E-07         |                     |
| Downregulated in hmt1\(^\Delta\) |                      |                  |                     |
| HMT1     | hnRNP arginine N-methyltransferase  | 2676.9           | 2.8                 |
|          |                                     | -1030            | -10.02              |
|          |                                     | 5.38E-54         |                     |
|          |                                     |                  |                     |
| YGL118C  | Putative uncharacterized protein     | 18.3             | 0.3                 |
|          |                                     | -61.0            | -5.86               |
|          |                                     | 3.80E-06         |                     |
|          |                                     |                  |                     |
| PHO12    | Repressible acid phosphatase        | 788.9            | 136.8               |
|          |                                     | -5.78            | -2.53               |
|          |                                     | 8.11E-23         |                     |
|          |                                     |                  |                     |
| SPL2     | Putative CDK inhibitor               | 74.6             | 12.7                |
|          |                                     | -5.87            | -2.55               |
|          |                                     | 6.13E-10         |                     |
|          |                                     |                  |                     |
| PHO11    | Repressible acid phosphatase        | 383.4            | 69.1                |
|          |                                     | -5.55            | -2.47               |
|          |                                     | 2.67E-19         |                     |
|          |                                     |                  |                     |
| IMD2     | Inosine-5'-monophosphate dehydrogenase | 1439.1     | 294.0               |
|          |                                     | -4.89            | -2.29               |
|          |                                     | 1.69E-26         |                     |
|          |                                     |                  |                     |
| YIR042C  | Uncharacterized protein              | 177.6            | 47.8                |
|          |                                     | -3.72            | -1.89               |
|          |                                     | 2.61E-10         |                     |
|          |                                     |                  |                     |
| PHO89    | Phosphate permease                  | 68.5             | 25.1                |
|          |                                     | -2.73            | -1.45               |
|          |                                     | 5.12E-03         |                     |
|          |                                     |                  |                     |
| YER188W  | Putative uncharacterized protein     | 99.5             | 39.2                |
|          |                                     | -2.54            | -1.34               |
|          |                                     | 1.75E-02         |                     |
|          |                                     |                  |                     |
| PHO5     | Repressible acid phosphatase        | 423.2            | 209.8               |
|          |                                     | -2.06            | -1.01               |
|          |                                     | 1.24E-03         |                     |
|          |                                     |                  |                     |
| VTC3     | Vacuolar transporter chaperone       | 1259.4           | 668.9               |
|          |                                     | -1.88            | -0.91               |
|          |                                     | 7.93E-02         |                     |
|          |                                     |                  |                     |
| FDC1     | Ferulic acid decarboxylase          | 654.5            | 390.7               |
|          |                                     | -1.68            | -0.74               |
|          |                                     | 5.38E-02         |                     |
|          |                                     |                  |                     |
| YHR214C-B| Transposon Ty1-H Gag-Pol polyprotein | 866.9            | 526.4               |
|          |                                     | -1.65            | -0.72               |
|          |                                     | 5.38E-02         |                     |

\(^a\)Mean normalized counts. \(^b\)p values adjusted with the Benjamini–Hochberg procedure are as detailed in the DESeq package (36).
ground, to produce a lys2Δ/arg4Δ/hmt1Δ strain. A reciprocal SILAC design integrating an isotope label swap was used. Four parallel cultures (Fig. 2; each of the background and hmt1/H9004 strains in heavy and in light media) were grown to mid-log phase. We had previously determined that there was no discernible difference in growth rates between hmt1/H9004 and wild-type (supplemental Fig. S2). After harvest and lysis, protein extracts were combined and subjected to separation by SDS-PAGE. This produced two sets of biological replicates, A and B. We confirmed the completeness of metabolic labeling by analyzing single slices of light-labeled, heavy-labeled, and 1:1 mixed labeled lysates, using LC-MS/MS and MaxQuant. Heavy-labeled lysate showed isotope incorporation of ~97% (heavy peak intensity divided by light peak intensity, supplemental Fig. S3).

LC-MS/MS analysis was performed on an Orbitrap mass spectrometer with one iteration of exclusion list analysis. After MaxQuant (45) processing, there were ~1,900 proteins identified, and quantitative information attained for ~1,500 proteins. We subjected the quantitative data set to filtering prior to statistical analysis; the rules used for data filtering were as in the Experimental Design and Statistical Rationale. Postfiltering, mean protein ratios were recalculated for the remaining 1,375 proteins, and B series ratios were inverted to match the A series ratios (i.e. a B ratio of 0.5 was converted to 2.0). Protein ratios for both series had distributions (supplemental Fig. S4) typical of SILAC analyses (62, 63). Statistically, we found 32 proteins to be of differential abundance between the background and hmt1Δ strains, where p < 0.05. Of the 32 proteins, 13 were of increased abundance in hmt1Δ whereas 19 showed decreased abundance (Table II). Interestingly, VTC3/Vtc3p were the only identical gene/protein pair that both showed significant downregulation. A lack of overlap in the genes/proteins of interest is not unexpected, given that the correlation between protein and mRNA levels can be poor (64). Several proteins of interest were unlikely to be detected by whole cell proteomics; for example, Pho5p, Pho11p and Pho12p are secreted extracellular acid phosphatases (65).

We investigated if there were functional relationships between the 32 differentially abundant proteins in hmt1Δ yeast (Table II). The GOMiner tool (47) was used to analyze these proteins, relative to a background of all identified proteins postfiltering. We found a significant enrichment of four functionally related categories. The category of phosphate metabolic process was enriched among the differentially abundant proteins in hmt1Δ (Fig. 1B), including the vacuolar transporter chaperone complex proteins (Vtc1p, Vtc3p, Vtc4p), the acid phosphatase Pho3p and the high-affinity phosphate transporter Pho84p (19, 59, 60). The category of transmembrane transporter activity also contained proteins associated with phosphate metabolism; Pho8p is a phosphatase (66) whereas Mir1p is a mitochondrial phosphate transporter (67). We noted that the vacuolar transporter chaperone complex proteins mapped to multiple categories, additionally being present in
the enriched cellular compartments of vacuolar membrane and vacuolar transporter chaperone complex. A list of the differentially abundant proteins found in each enriched functional category is presented in supplemental Table S5.

Phosphate regulation and metabolism is controlled by the PHO (phosphate-responsive signaling) pathway in yeast (68). The vacuolar transporter chaperone (VTC) proteins are known to be induced under low-phosphate conditions, as are the Pho84p phosphate transporter and the Pho8p vacuolar phosphatase (19). All are under the control of the Pho4p transcriptional activator. The fact that these PHO-regulated proteins uniformly displayed a decreased abundance in hmt1Δ suggests PHO pathway repression, as was also seen in the gene expression analysis. It is notable that three of the four proteins of the VTC complex (Vtc1p, Vtc3p, Vtc4p) demonstrated decreased abundance in hmt1Δ. The VTC complex plays a crucial role in phosphate homeostasis as part of the PHO pathway (19, 69, 70). The VTC complex is present on the endoplasmic reticulum, at vacuoles, and the cell periphery, but is enriched at the vacuolar membrane (71). The Emp70p and Pho8p proteins, significantly downregulated as per Vtc1p, Vtc3p, Vtc4p, also localize to this cellular component (72, 73).

### Table II

| Protein | Uniprot | Description | Mean ratio | Peptides (unique) | p value | Coverage |
|---------|---------|-------------|------------|-------------------|---------|----------|
| **Upregulated in hmt1Δ** | | | | | | |
| Psp2p | P50109 | Uncharacterized protein | 1.99 | 6 (6) | 1.85E-02 | 15.3% |
| Scw4p | P53334 | Probable family 17 glucosidase | 1.78 | 2 (2) | 1.42E-02 | 8.5% |
| Twf1p | P53250 | Twinfilin-1 | 1.72 | 2 (2) | 1.39E-02 | 9.0% |
| Arb1p | P40024 | ABC transporter ATP-binding protein | 1.58 | 20 (20) | 3.40E-02 | 34.8% |
| Gar1p | P28007 | H/ACA ribonucleoprotein complex subunit 1 | 1.48 | 2 (2) | 1.29E-02 | 17.1% |
| Arg5,6p | Q01217 | Protein ARG5,6, mitochondrial | 1.41 | 22 (22) | 2.14E-02 | 38.9% |
| Cmk1p | P27466 | Calcium/calmodulin-dependent protein kinase I | 1.39 | 4 (4) | 2.12E-02 | 10.7% |
| Cve4p | Q04201 | CUE domain-containing protein | 1.38 | 3 (3) | 2.57E-02 | 41.9% |
| Mtr1p | P23641 | Mitochondrial phosphate carrier protein | 1.35 | 8 (8) | 2.91E-02 | 43.7% |
| Atp17p | Q06405 | ATP synthase subunit f, mitochondrial | 1.34 | 5 (5) | 4.76E-02 | 33.7% |
| Dbp2p | P24783 | ATP-dependent RNA helicase | 1.31 | 22 (22) | 4.16E-02 | 43.6% |
| Bzz1p | P38822 | Uncharacterized protein | 1.3 | 3 (3) | 4.86E-02 | 8.4% |
| YJL068C | P40363 | S-formylglutathione hydrolase | 1.3 | 4 (4) | 4.57E-02 | 22.1% |
| **Downregulated in hmt1Δ** | | | | | | |
| Vtc3p | Q02725 | Vacular transporter chaperone | 0.24 | 13 (13) | 1.21E-03 | 18.1% |
| Pho84p | P25297 | Inorganic phosphate transporter | 0.3 | 3 (3) | 2.27E-02 | 7.3% |
| Vtc1p | P40046 | Vacular transporter chaperone | 0.52 | 3 (3) | 9.63E-03 | 14.7% |
| Vtc4p | P47075 | Vacular transporter chaperone | 0.57 | 17 (17) | 1.38E-02 | 23.2% |
| Emp70p | P32802 | Transmembrane 9 superfamily member 1 | 0.61 | 2 (2) | 2.71E-02 | 6.3% |
| Pho8p | P11491 | Repressible alkaline phosphatase | 0.63 | 7 (7) | 1.56E-02 | 19.3% |
| YLR413W | Q06689 | Cell membrane protein | 0.66 | 4 (4) | 1.06E-02 | 5.6% |
| Pho3p | P24031 | Constitutive acid phosphatase | 0.69 | 5 (5) | 3.93E-02 | 14.6% |
| Fpr4p | Q06205 | FK506-binding protein 4 | 0.7 | 9 (9) | 3.89E-02 | 30.2% |
| Pdr5p | P33302 | Pleiotropic ABC efflux transporter of multiple drugs | 0.72 | 14 (11) | 3.23E-02 | 10.1% |
| Hem13p | P11353 | Oxygen-dependent coxproporphyrinogen-III oxidase | 0.73 | 4 (4) | 4.97E-02 | 15.2% |
| Fsh1p | P38777 | Family of serine hydrolases 1 | 0.73 | 6 (6) | 2.35E-02 | 34.0% |
| Bat2p | P47176 | Branched-chain-amino-acid aminotransferase, cytosolic | 0.73 | 11 (8) | 3.40E-02 | 27.4% |
| Lsb5p | P25369 | LAS seventeen-binding protein 5 | 0.74 | 2 (2) | 3.41E-02 | 8.5% |
| Lrg1p | P35688 | Rho-GTPase-activating protein | 0.75 | 3 (3) | 3.62E-02 | 3.6% |
| YJR029W* | P47100 | Transposon Tyn1-JR2 Gag-Pol polyprotein | 0.75 | 45 (0) | 3.17E-02 | 33.4% |
| Lac1p | P28496 | Sphingosine N-acyltransferase | 0.75 | 3 (3) | 4.44E-02 | 12.0% |
| Dbp10p | Q12389 | ATP-dependant RNA helicase | 0.76 | 3 (3) | 3.72E-02 | 4.8% |
| Hxt3p | P32466 | Low-affinity glucose transporter | 0.77 | 6 (5) | 4.31E-02 | 12.6% |

*Peptides common to Q12414; Q03612; P0C2I3; P0C2I5; P0C2I7; Q04214; Q12490; P0C2I9; P47100; P0C2J0; Q04670; P0C2I2; Q04711; Q92393; O13535; Q03619; Q12088; Q12316; Q12193.

*Uniprot accession number of protein. **Average (mean) ratio of both A and B replicates. "Peptides identified from protein, unique peptides in brackets. *"Peptides with abundance differences of p < 0.05, Bayes-moderated. **Peptide sequence coverage of identified protein.

### Supplemental Table S5

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dance in hmt1Δ yeast (Table II). Three of these 13 proteins were of interest as they are either known or putative substrates of the methyltransferase Hmt1p (4), however it is notable that there was no significant increase or decrease in transcript levels of these or any other known or putative Hmt1 substrate. Gar1p has been shown to be methylated by Hmt1p (5, 74), whereas Psp2p and Dbp2p have been proposed as substrates of Hmt1p because of the presence of RGG-rich regions (75). To further investigate the increase in abundance of Hmt1p substrates, we subjected yeast strains carrying TAP-tagged Gar1p and Dbp2p to HMT1 knockout. One-dimensional SDS-PAGE and immunoblotting validated an increased abundance of Dbp2p in hmt1Δ (supplemental Fig. S5). This suggests the regulation of Dbp2p abundance at the protein level. However, Gar1p in hmt1Δ yeast was found to migrate anomalously because of the absence of arginine methylation, appearing as a band of lower mass (supplemental Fig. S5).

Network Analysis Reveals No Associations Between Known Hmt1p Substrates and Phosphate Regulation—It was not immediately apparent how deletion of HMT1 could affect the regulation of phosphate in the yeast cell. No known Hmt1p substrates showed any differences in gene expression, whereas the above-mentioned Dbp2p, Gar1p and Psp2p proteins have no known association with the regulation of phosphate. Given that arginine methylation can modulate protein-protein or protein-RNA interactions (52, 76) we first examined networks of physical and functional interactions in the STRING database (77) for evidence of interactions between known substrates of Hmt1p and the differentially expressed genes/differentially abundant proteins. As Hmt1p is known to methylate many RNA-binding heterogeneous nuclear ribonucleoproteins (hnRNPs), it was not surprising that several of our differentially expressed transcripts have been reported to interact with known Hmt1p substrates Nab2p and Sbp1p (supplemental Fig. S6). However, Nab2p is a general RNA-binding protein, involved in nuclear export of over 2500 mRNAs (78). Similarly, Sbp1p has been reported to interact with >1000 different RNAs (79). These interactions are therefore not specifically associated with phosphate regulation. Though not revealed in the STRING analysis, several of our differentially expressed genes/differentially abundant proteins have been reported to interact with Hmt1p substrates. Siz1p has been reported to interact physically with Nab2p and Tif4632p, another putative substrate of Hmt1p (81). However, there is no evidence of those interactions being methylation-dependent. Interestingly, transcripts YFL067W and YHR214W-A have been reported to bind Hek2p (82). Although Hek2p is not known to be a substrate of Hmt1p, or reported to carry arginine methylation in yeast, its human homolog hnRNP K has been reported to be methylated at five sites by PRMT1, the human equivalent of Hmt1p (83). Notwithstanding these observations, there was no apparent functional association between phosphate regulation and known substrates of Hmt1p that had differentially expressed genes or differentially abundant proteins.

HMT1 Knockout Yeast Shows Decreased Acid Phosphatase Activity and Lower Total Pi Levels—During mid-log growth, hmt1Δ showed a decrease in expression or abundance of genes and proteins associated with phosphate regulation and metabolism (Tables I and II, Fig. 1). These included repressible acid phosphatase family proteins (PHOS, PHO11, and PHO12), high-affinity phosphate transporters (Pho84p and PHO89), subunits of the VTC complex associated with polyP accumulation (Vtc1p, Vtc3p, and Vtc4p), and SPL2 which has whose product affects the localization of the Pho87p low affinity phosphate transporter (84). Accordingly, we investigated whether there was any change in extracellular acid phosphatase activity, along with total Pi, inorganic polyphosphate (polyP) stores between hmt1Δ and wild-type yeast.

To assay extracellular acid phosphatases, wild-type and hmt1Δ cells were first grown in YEPD and then conditioned in phosphate depleted (MM-KCl) or phosphate replete (MM-K2HPO4) media. Cells were then washed prior to the pNPP hydrolysis assay. Compared with the wild type, hmt1Δ showed a ~33% reduction (p < 0.05) in pNPP hydrolysis when cells were first conditioned in either phosphate depleted or phosphate replete media (Fig. 3A). This indicates a significant decrease in extracellular acid phosphatase activity. A longer conditioning also showed a similar trend (supplemental Fig. S7). These observations validated the gene expression results (Table I), in which hmt1Δ showed a significant down-regulation in the genes of secreted acid phosphatases PHO5, PHO11, and PHO12. To determine that the loss of enzymatic activity of Hmt1p was the cause of the decreased acid phosphatase activity, we also assayed a strain where we had engineered the G68R mutation into the chromosomal gene for Hmt1p. This mutation generates an inactive version of the enzyme (52, 85). The inactive G68R Hmt1p showed similar decreases in extracellular acid phosphatase activity as the hmt1Δ strain (Fig. 3A), indicating that loss of Hmt1p activity and not just the loss of the Hmt1p protein itself causes a decreased level of extracellular acid phosphatases.

Total P_i was extracted and quantified from wild-type and hmt1Δ cells grown in phosphate depleted, phosphate replete or YEPD media (Fig. 3B). The hmt1Δ cells showed a significant 30% reduction (p < 0.0001) of total P_i levels, as compared with wild-type, when both were grown in phosphate depleted media. This likely reflects the lower abundance of the high affinity phosphate transporter Pho84p in hmt1Δ, as seen in the proteomic analysis (Table II). Differences in response to different media were also seen, in that wild-type cells showed a significant 30% decrease (p < 0.0001) in total P_i when grown in phosphate replete MM-KH2PO4 medium compared with phosphate depleted MM-KCl. In contrast, hmt1Δ cells exhibited no significant changes in P_i levels between phosphate replete and depleted media. No differences
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in Pi levels were found between wild-type and hmt1Δ grown in YEPD (which has a higher concentration of phosphate than MM-KCl and has higher nutrients).

Finally, polyP abundance between wild-type and hmt1Δ cells was examined when cells were subcultured from YEPD to different media; this is known to affect polyP synthesis and accumulation (86). Accordingly, we compared the polyP accumulation between wild-type and hmt1Δ that were first grown in YEPD and then subcultured to either phosphate depleted, phosphate replete or YEPD media. polyP accumulation was examined at the lag, log, stationary and overnight stages of growth (Fig. 3C and supplemental Fig. S8), by enzymatic hydrolysis of polyP to Pi with purified exopolyphosphatase Ppx1p. Upon transfer of cells to phosphate depleted medium (MM-KCl), we observed low overall polyP levels and little accumulation of polyP from lag through to stationary phase in wild-type or hmt1Δ (86). However, compared with the wild-type, hmt1Δ showed a significant decrease in polyP levels at the log phase (p = 0.0023). Upon transfer of cells from YEPD to phosphate replete medium (MM-KH2PO4), successful accumulation of polyP was evident in wild-type and hmt1Δ cells (as seen by overall higher Pi, as compared with MM-KCl). This is consistent with previous reports (87). Despite the downregulation of VTC genes and proteins in hmt1Δ cells (Tables I and II), which are involved in polyP synthesis (19), a significant increase in polyP concentration in hmt1Δ was observed compared with the wild-type after growth overnight (p = 0.0464). Upon transfer of cells from YEPD to fresh YEPD, wild-type and hmt1Δ cells from lag to stationary phase showed an increased increase in polyP levels (86). Contrary to VTC regulation (Tables I and II), however, a significant increase in polyP concentration was observed in hmt1Δ compared with the wild-type in the lag (p < 0.0005) and log (p = 0.0361) phases.

Pho4p Is An In Vitro Substrate of Hmt1p with A Single Arginine Methylation Site at Arg-241—Our studies of gene and protein expression in hmt1Δ, and the analysis of extracellular phosphatases and intracellular Pi, highlighted a dysregulation in phosphate homeostasis. Given that many of the genes are regulated by transcription factor Pho4p, which undergoes nucleocytoplasmic shuttling, and given that Hmt1p methylation can mediate nuclear exit of proteins (88, 89), we investigated whether Pho4p is a substrate of Hmt1p. We also examined whether Pho2p, which complexes with Pho4p for activation of gene expression, is a substrate of Hmt1p.

Recombinant Hmt1p, Pho2p and Pho4p were purified from E. coli and used in in vitro methylation assays. Purified Npl3p, a known substrate of Hmt1p (90), was used as a positive

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**Fig. 3.** The hmt1Δ mutant shows dysregulation of phosphate metabolism. A, The hmt1Δ mutant has significantly lower extracellular acid phosphatase activity compared with wild-type, after 2 h’s conditioning in phosphate depleted (MM-KCl) or phosphate replete (MM-KH2PO4) media. Assay results are at time 180 min, after adding cells to the pNPP reaction mix. B, The hmt1Δ mutant shows lower total Pi compared with wild-type in phosphate depleted medium (MM-KCl). No differences in total P, were observed for hmt1Δ between low (MM-KCl) and high phosphate (MM-KH2PO4) media, however the wild-type did show a significant decrease between these two conditions. C, The hmt1Δ mutant, compared with wild-type, shows significant changes in polyP levels at some phases of growth in MM-KCl, MM-KH2PO4 and YEPD. Time 3 h is lag, 7 is log, 10 is stationary and 25 h is overnight. Data information: Data are presented as mean and error bars indicate standard deviation. Mean was obtained from at least two biological replicates. In A, B and C, * indicates p < 0.05, ** indicates p < 0.002, *** indicates p < 0.0005 and **** indicates p < 0.0001 (Student’s unpaired t test).
We investigated the Arg-241 monomethylation site in vivo, for its presence and possible function when mutated to lysine. Overexpressed, His-tagged Pho4p from wild-type yeast was purified and analyzed by ETD-MS/MS. Unexpectedly, this did not reveal the presence of arginine methylation at Arg-241 (supplemental Fig. S9). A chromosomal GFP fusion of Pho4p (29) was also immunoprecipitated and analyzed by MS/MS. Although expressed at native abundance, this also did not reveal the presence of methylation at Arg-241, although it should be noted that the peptides covering this region of Pho4p were detected at low levels (supplemental Fig. S10). Phosphosites at Ser-242 and Set-243 were, however, confirmed (supplemental Figs. S9 and S10). Finally, we mutated Arg-241 to lysine in the chromosomal pho4 gene and assayed extracellular acid phosphatase levels compared with wild type. This was done in MM-KCl and in MM-KH₂PO₄. This revealed a dysregulation of extracellular phosphatase activity (supplemental Fig. S11) although as a significant increase compared with wild type (p < 0.05) in MM-KCl. This was unexpected as a significant decrease in activity was seen in hmt1Δ cells under the same conditions (Fig. S4).

HMT1 Knockout Does Not Affect Nuclear Localization of Pho4p Under Phosphate Limitation—It is known that Pho4p multimerizes with Pho2p to activate the transcription of genes involved in the PHO regulatory pathway (91). Pho4p is localized to the nucleus and the cytoplasm in phosphate depleted and phosphate rich media, respectively (92). The shuttling of this transcription factor is dependent on its phosphorylation state, where the hypophosphorylation of Pho4p is critical to its nuclear localization (93). By contrast, Pho2p is localized in the nucleus and does not shuttle (94). As noted above, arginine methylation is known to be involved in the nucleocytoplasmic transport of Hmt1p substrates. The shuttling of Npl3p and Nab2p between the nucleus and the cytoplasm is methylation dependent (85, 88, 89, 95). Hence, we investigated whether the localization of Pho4p, being an in vitro substrate of Hmt1p, is affected by the loss of arginine methylation.

A strain containing chromosomally GFP-tagged Pho4p, previously used for the study of Pho4p localization (29), and one containing GFP-tagged Pho2p (96) were subjected to deletion of HMT1. GFP-tagged Tef1p (96) was used as a cytoplasmic localization control. We studied the localization of Pho2p and Pho4p fusion proteins in phosphate replete and depleted media. In both the wild-type and hmt1Δ mutant, there was no difference in Pho4p localization in either condition. Pho4p-GFP localized in the cytoplasm under replete phosphate and localized to the nucleus upon phosphate depletion (Fig. 6). Statistically, there was no significant difference in the level of Pho4p-GFP nuclear localization between the wild-type and knockout mutant (supplemental Fig. S12). As expected, the localization of Pho2p-GFP was nuclear in the wild-type and hmt1Δ mutant, whereas the localization of the control protein translational elongation factor Tef1p-GFP was cytosolic.
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Fig. 5. Hmt1p methylates Pho4p in vitro at Arg-241. A, Protein sequence of Pho4p. The peptide carrying the mono-methylarginine site at position 241, discovered by ETD-MS/MS, is underlined and in bold blue. All other Pho4p peptides detected by ETD-MS/MS are blue. Regions shown in black were not detected in ETD-MS/MS; these included very large tryptic peptides at positions 41 to 79 (mass of 4520.8 Da), position 80 to 110 (mass of 3494.6 Da) and position 160 to 188 (mass of 3028.7 Da). Phosphorylation sites reported in the literature are shown in red.
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![Figure 6: Deletion of HMT1 has no effect on the nuclear localization of Pho4p-GFP under phosphate limited conditions compared with the wild-type. Pho2p-GFP was localized to the nucleus, even with the deletion of HMT1, whereas Tef1p-GFP was localized in the cytoplasm. Images were taken at 100x objective (oil immersion).](image)

DISCUSSION

Here we report that phosphate homeostasis in S. cerevisiae is disrupted through the deletion of HMT1. This was seen at both a transcriptomic and proteomic level; on deletion of HMT1, we observed a significant decrease in expression and abundance of many Pho4p target genes and their protein products. These included the genes PHO5, PHO11, PHO12, SPL2, PHO89, and VTC3 (Table I) and proteins Pho3p, Pho8p, Pho84p, Vtc1p, Vtc3p, and Vtc4p (Table II) (19). Functionally, we found that these changes were associated with a reduction in the activity of component(s) of PHO regulation, especially but not only the decrease in extracellular acid phosphatase activity (Fig. 3A). We showed that this phenotype was also seen in a G68R mutant of Hmt1p, showing that the loss of Hmt1p activity, and not just a loss of the entire protein, leads to dysregulation of phosphate homeostasis. A decrease in total P_i levels was seen in hmt1Δ in low phosphate conditions (Fig. 3B). By contrast, the concentration of total P_i was similar between hmt1Δ and wild type in high phosphate media MM-KH_2PO_4 or in YEPD, which could reflect the accumulation of phosphate in cells through passive diffusion (97). Interestingly, although significant changes in polyP levels were observed in hmt1Δ compared with wild type (Fig. 3C), these were contrary to those expected, being a decrease in polyP concentrations in phosphate replete media YEPD and MM-KH_2PO_4 and increase in the phosphate depleted medium MM-KCl (86). This may be because of the VTC complex having functions separate to polyphosphate metabolism, such as vacuolar membrane fusion, V-ATPase activity and microautophagy (70, 71, 98).

In vitro investigation into Pho4p revealed that it could be monomethylated at arginine 241 by Hmt1p, in an RSS motif (Fig. 4 and 5). The motif is different to the canonical RGG motif for Hmt1p; noncanonical sites of Hmt1p methylation have been reported elsewhere (10, 11) although these too have arisen from in vitro experiments. Our analysis of Pho4p methylation in vivo was inconclusive as we did not detect methylation at Arg-241 or any other site, whether on a natively expressed GFP Pho4p fusion or on overexpressed Pho4p. However, it is possible that Arg-241 methylation only occurs under specific conditions, or is of low stoichiometry, and thus was undetected here. It is also possible that other regulatory relationships exist between arginine methylation and phosphorylation homeostasis in S. cerevisiae cell; a recent study reported the presence of polyphosphate on protein Nsr1p (99) which is a known in vivo substrate of Hmt1p (5).

The localization of some Hmt1p substrate proteins is known to be affected by methylation, including Npl3p, Hrb1p, Nab2p and Gbp2p (16, 74, 85, 89, 100). Knowing this, we investigated whether the loss of HMT1 could affect the localization of Pho4p, interfering with the Pho4p nucleocytoplasmic shuttling that is dependent on its degree of phosphorylation (101). Using chromosomal GFP-fusions, we detected no change in the subcellular localization of Pho4p in the absence of HMT1 (Fig. 6). This suggests that methylation of Pho4p, if confirmed in vivo, must affect phosphate regulation through mechanism(s) that affect the transcriptional activity but not localization of Pho4p.

The location of the putative arginine methylation site in Pho4p may provide clues to its function. There are two possible models for this. The first is that arginine methylation acts to increase the homodimerization of Pho4p or its binding affinity to DNA. To activate transcription of its target genes, Pho4p binds DNA as a homodimer with its basic helix-loop-helix (bHLH) motif, located at the C terminus (102). The methylation site at Arg-241 is located between the oligomerization and bHLH DNA-binding domains of Pho4p (Fig. 7). Based on the C-terminal crystal structure of Pho4p (103), the methylarginine is unlikely to be within the DNA-binding region. However, the proximity of Arg-241 to the oligomerization

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B. The annotated ETD-MS/MS spectra for the methylated Pho4p peptide R<sub>met</sub>SSGALVDDDKR from the in vitro methylation assay (top) and the synthetic methylated peptide (bottom) show near-identical fragmentation patterns, validating the identification of this methylarginine site on Pho4p. Summarized ion fragment coverages, where c- and z-ions and their derivatives are shown in each spectra. Precursor and charge-reduced precursor ions - c- and z-ions, which are prominent ions resulting from -NH_3 and methylarginine-associated losses - and their measured masses are labeled in the spectra. Methylarginine-associated neutral losses are abbreviated as follows: monomethylamine (MMA), monomethylguanidine (MMG). Losses from NH_3 are shown as "'".
and bHLH domains means its methylation could be involved in mediating the protein-protein interactions necessary for the activation of the target genes of Pho4p. There is a dimerization precedent in hnRNP protein Npl3p, where nuclear Hmt1p-mediated arginine methylation increases self-dimerization by \( \sim 2\)-fold (52, 104). The dimerization domain of Npl3p is in the C-terminal SR-domain, from positions 276–364, and loss of Npl3p dimerization results in defects in translation (105). We hypothesize that the function of the putative arginine methylation site on Pho4p could be like Npl3p, potentially increasing its homodimerization. If this is the case Pho4p could still dimerize in the absence of arginine methylation, albeit with lower affinity. This model could explain the significant reduction, but not complete loss of expression, of Pho4p target genes and proteins in hmt1Δ.

A second model for the putative Arg-241 methylation in Pho4p involves the interplay between phosphorylation and methylation. Interplay between modifications is emerging as a widespread means for the regulation of protein function, including in protein interaction codes (106). Pho4p is phosphorylated at eight different serine residues, five of which are phosphorylated by the Pho80p-Pho85p cyclin-CDK complex under high-phosphate conditions (92, 93). Four of these five phosphosites are well characterized in their respective roles: phosphorylation of Ser-114 and -128 signals nuclear export; phosphorylation of Ser-152 decreases nuclear import; and phosphorylation of Ser-223 decreases the binding affinity for Pho2p. The remaining three phosphoserines at positions 204, 242, and 243 are not phosphorylated by Pho85p, and their kinase(s) and molecular functions are unknown (107–109). It is noteworthy that the methylarginine site on Pho4p, reported in this study, is directly adjacent to the phosphoserines at positions 242 and 243 (Fig. 7) (109). Given its proximity to the DNA-binding domain, arginine methylation of Pho4p could potentially serve as a recruitment signal for transcriptional machinery, or to recruit kinases for the phosphorylation of Ser-242 and -243. A similar example involves the methylation of transcription factor STAT6 at Arg-27 (110), where the loss of arginine methylation leads to a decrease in IL4-dependent phosphorylation. Alternatively, given that all of the characterized phosphoserines in Pho4p inhibit its transcriptional activity (93), Pho4p methylation could serve to block this inhibition. In human cells, the methylation of Arg-296 and -299 by PRMT1 inhibited the phosphorylation of Ser-302 on hnRNP K, involved in chromatin remodelling, transcription, RNA splicing, mRNA stability and translation (111). Loss of methylation on these arginine residues led to an increase of p53-independent apoptosis upon DNA damage.

Post-translational modification of proteins, or combinations thereof, are important ways by which nutrient sensing can be controlled. Examples include the PHO pathway itself and, in mammalian cells, the interplay between O-linked β-N-acetyl glucosamine (O-GlcNAc) and phosphorylation (112). Our research has highlighted a possible new link between phosphate regulation and SAM levels, where SAM is the major methyl donor inside the cell. SAM is synthesized alongside phosphate and diphosphate from methionine and ATP by the S-adenosylmethionine synthetases Sam1p and Sam2p. Interestingly, the intracellular concentrations of SAM and phosphate are known to be correlated. The accumulation of SAM in the adenosine kinase mutant ado1Δ was accompanied by an upregulation in phosphate related transcripts and increased cellular concentrations of phosphate and polyP (113). Furthermore, polyP contributes to stability of SAM (113). The downregulation of the PHO regulon upon deletion of HMT1, revealed in our study, suggests an interdependency between the regulation of SAM and phosphate levels. Simplistically, this may be a means by which the cell does not expend resources on phosphate sequestration if there is not sufficient SAM to undertake a large range of other metabolic activities. This donor sensing and regulation of protein activity is like the dependence of acetyl-CoA donor levels on the activity of lysine acetyltransferases (KATs), which acetylate enzymes that regulate various metabolic processes in the cell (114). In conclusion, this study has shown that loss of Hmt1p-mediated arginine methylation leads to the dysregulation of phosphate homeostasis. Although the monomethylation of Pho4p at Arg-241 may play a role in this process, this was not validated in vivo and thus the exact molecular mechanisms underlying dysregulation remain to be confirmed.
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DATA AVAILABILITY

Data from SILAC experiments has been submitted to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (57) with the data set identifier PXD004054. MaxQuant output for SILAC experiments are available as supplemental Data S1 and S2. Annotated MS/MS spectra can be visualised by using MS-Viewer (http://msviewer.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msviewer) with the search keys: 10lc8v6ekm (data set A) and 0gyw6hg1 (dataset B). RNA-Seq reads have been deposited in the NCBI sequence read archive (SRA) under the accession number SRP072252. Microarray data have been deposited in the NCBI Gene Expression Omnibus under the accession number GSE99869 (58).

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REFERENCES

1. Ong, S.-E., Mittler, G., and Mann, M. (2004) Identifying and quantifying in vivo methylation sites by heavy metal SILAC. Nat. Methods 1, 119–126
2. Gary, J. D., Lin, W.-J., Yang, M. C., Herschman, H. R., and Clarke, S. (1996) The predominant protein-arginine methyltransferase from Schizosaccharomyces cerevisiae. J. Biol. Chem. 271, 12585–12594
3. Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, N., O’Shea, E. K., and Weissman, J. S. (2003) Global analysis of protein expression in yeast. Nature 425, 737–741
4. Low, J. K. K., Hart-Smith, G., Erce, M. A., and Wilkins, M. R. (2013) Analysis of the proteome of Saccharomyces cerevisiae for methylarginine. J. Proteome Res. 12, 3884–3899
5. Yagoub, D., Hart-Smith, G., Moecking, J., Erce, M. A., and Wilkins, M. R. (2013) Yeast proteins Gap1p, Nop1p, Npl3p, Npl1p, and Rps2p are natively methylated and are substrates of the arginine methyltransferase Hmt1p. Proteomics 13, 3209–3218
6. Hart-Smith, G., Yagoub, D., Tay, A. P., Pickford, R., and Wilkins, M. R. (2016) Large scale mass spectrometry-based identifications of enzyme-mediated protein methylation are subject to high false discovery rates. Mol. Cell. Proteomics 15, 989–1006
7. Plank, M., Fischer, R., Geoghegan, V., Charles, P. D., Konietzny, R., Acuto, O., Pears, C., Schofield, C. J., and Kessler, B. M. (2015) Expanding the yeast protein arginine methylome. Proteomics 15, 3232–3243
8. Kuo, M. H., Xu, X. J., Bolick, H. A., and Guo, D. (2009) Functional connection between histone acetyltransferases Gcn5p and histone methyltransferase Hmt1p. Biochem. Biophys. Acts 1789, 395–402
9. Lacoste, N., Utley, R. T., Hunter, J. M., Poinier, G. G., and Côté, J. (2002) Disruptor of telomeric silencing-1 is a chromatin-specific histone H3 histone methyltransferase. J. Biol. Chem. 277, 30421–30424
10. Low, J. K. K., and Wilkins, M. R. (2012) Protein arginine methylation in Saccharomyces cerevisiae. FEBS J. 279, 4423–4443
11. Low, J. K. K., Im, H., Erce, M. A., Hart-Smith, G., Snyder, M. P., and Wilkins, M. R. (2016) Protein substrates of the arginine methyltransferase Hmt1 identified by proteome arrays. Proteomics 16, 465–476
12. Wei, H., Mundade, R., Lange, K., and Lu, T. (2014) Protein arginine methylation of non-histone proteins and its role in diseases. Cell Cycle 13, 32–41
13. Yu, Z., Chen, T., Hébert, J., Li, E., and Richard, S. (2009) A mouse PRMT1 null allele defines an essential role for arginine methylation in genome maintenance and cell proliferation. Mol. Cell Biochem. 29, 2992–2996
14. Wei, H., Mundade, R., Lange, K., and Lu, T. (2014) Protein arginine methylation of non-histone proteins and its role in diseases. Cell Cycle 13, 32–41
15. Pavliak, M. R., Scherer, C. A., Chen, J., Roshon, M. J., and Ruley, H. E. (2000) Arginine N-methyltransferase 1 is required for early postimplantation mouse development, but cells deficient in the enzyme are viable. Mol. Cell. Biol. 20, 4859–4869
16. Yu, M. C., Bachand, F., McBride, A. E., Korn, S., Casolari, J. M., and Silver, P. A. (2004) Arginine methyltransferase affects interactions and recruitment of mRNA processing and export factors. Genes Dev. 18, 2024–2035
17. Yu, M. C., Lamming, D. W., Eskin, J. A., Sinclair, D. A., and Silver, P. A. (2006) The role of protein arginine methylation in the formation of silent chromatin. Genes Dev. 20, 3249–3254
18. Milliman, E. J., Hu, Z., and Yu, M. C. (2012) Genomic insights of protein arginine methyltransferase 1 binding reveals novel regulatory functions. BMC Genomics 13, 728–728
19. Ogawa, N., DeRisi, J., and Brown, P. O. (2000) New components of a system for phosphate accumulation and polyphosphate metabolism in Saccharomyces cerevisiae revealed by genomic expression analysis. Mol. Biol. Cell 11, 4309–4321
20. Wykoff, D. D., and O’Shea, E. K. (2001) Phosphate transport and sensing in Saccharomyces cerevisiae. Genetics 155, 1491–1499
21. Hothon, M., Neumann, H., Lenherr, E. D., Wehner, M., Rybin, V., Hassa, P. O., Uttenweiler, A., Reinhardt, M., Schmidt, A., Seiler, J., Ladurner, A. G., Herrmann, C., Scheffzek, K., and Mayer, A. (2009) Catalytic core of a membrane-associated eukaryotic polyphosphate polymerase. Science 324, 513–516
22. Desfougeres, Y., Gerasimaitre, R. U., Jessen, H. J., and Mayer, A. (2016) Vtch, a novel subunit of the vacuolar transporter chaperone complex, regulates polyphosphate synthesis and phosphate homeostasis in yeast. J. Biol. Chem. 291, 22262–22275
23. Thomas, M. R., and O’Shea, E. K. (2005) An intracellular phosphate buffer filters transient fluctuations in extracellular phosphate levels. Proc. Natl. Acad. Sci. U.S.A. 102, 9565–9570
24. Moreno, S. N. J., and Docampo, R. (2013) Polyphosphate and its diverse functions in host cells and pathogens. PLoS Pathog. 9, e1003230
25. Kornberg, A., Rao, N. N., and Ault-Riche´ , D. (1999) Inorganic polyphosphate: a molecule of many functions. Annu. Rev. Biochem. 68, 89–125
26. Bru, S., Martinez-Lainez, J. M., Hernandez-Ortega, S., Quandt, E., Torres-Torronteras, J., Marti, R., Canadell, D., Arino, J., Sharma, S., Jimenez, J., and Clotet, J. (2016) Polyphosphate is involved in cell cycle progression and genomic stability in Saccharomyces cerevisiae. Mol. Microbiol. 101, 367–380
27. Zhang, L., Hamey, J. J., Hart-Smith, G., Erce, M. A., and Wilkins, M. R. (2014) Elongation factor methyltransferase 3–a novel eukaryotic lysine methyltransferase. Biochem. Biophys. Res. Commun. 451, 229–234
28. Janke, C., Magiera, M. M., Rathfelder, N., Taxis, C., Reber, S., Maekawa, H., Moreno-Borchart, A., Doenges, G., Schwob, E., Schiebel, E., and
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67. Phelps, A., Schobert, C. T., and Wohlrab, H. (1991) Cloning and characterization of the mitochondrial phosphate transport protein gene from the yeast Saccharomyces cerevisiae. Biochemistry 30, 248–252
68. Secco, D., Wang, C., Shou, H., and Whelan, J. (2012) Phosphate homeostasis in the yeast Saccharomyces cerevisiae, the key role of the SPX domain-containing proteins. FEBS Lett. 586, 289–295
69. Cohen, A., Perzov, N., Nelson, H., and Nelson, N. (1999) A novel family of yeast chaperons involved in the distribution of V-ATPase and other membrane proteins. J. Biol. Chem. 274, 28885–28893
70. Müller, O., Neumann, H., Bayer, M. J., and Mayer, A. (2003) Role of the Vtc proteins in V-ATPase stability and membrane trafficking. J. Cell Sci. 116, 1107–1115
71. Uttenweiler, A., Schwarz, H., Neumann, H., and Mayer, A. (2007) The yeast chaperons involved in the distribution of V-ATPase and other membrane proteins. FEBS Lett. 586, 289–295
72. Frankel, A., and Clarke, S. (1999) RNase treatment of yeast and mammalian cells. FEBS Lett. 459, 1–6
73. Klonowsky, D. J., and Emr, S. D. (1989) Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. EMBO J. 8, 2241–2250
74. Xu, C., Henry, P. A., Setya, A., and Henry, M. F. (2003) In vivo analysis of nucleolar proteins modified by the yeast arginine methyltransferase Hmt1/Rmt1p. Biochem. Biophys. Res. Commun. 295, 391–400
75. Chen, Y. C., Milliman, E. J., Goulet, L., Côté, J., Jackson Ca, Volbracht Ja Yu, M. C., and Cote, J. (2010) Protein arginine methyltransferase facilitates cotranscriptional recruitment of pre-mRNA splicing factors. Mol. Cell. Proteomics 10, 5245–5256
76. Jensen, L. J., Kuhn, M., Stark, M., Chaffron, S., Creevey, C., Muller, J., Uttenweiler, A., Schwarz, H., Neumann, H., and Mayer, A. (2007) The yeast transcriptome and a messenger ribonucleoprotein core structure. J. Biol. Chem. 284, 34911–34917
77. Mitchell, S. F., Jain, S., She, M., and Parker, R. (2013) Global analysis of yeast mRNPs. Nat. Struct. Mol. Biol. 20, 127–133
78. Deleted in proof.
79. Srikumar, T., Lewicki, M. C., and Raught, B. (2013) A global S. cerevisiae small ubiquitin-related modifier (SUMO) system interactome. Mol. Syst. Biol. 9, 668–668
80. Hasegawa, Y., Irie, K., and Gerber, A. P. (2008) Distinct roles for Khd1p in the localization and expression of bud-localized mRNAs in yeast. RNA 14, 2333–2347
81. Gietl, D., Buerger, A., Becker, U., Huh, W.-K., Falvo, J. V., and Gerke, L. C. (2012) Pho4 out of the nucleus: Pho4 out of the nucleus. J. Cell Biol. 195, 977–980
82. Azevedo, C., Livermore, T., and Salari, A. (2015) Protein polyphosphorylation of lysine residues by inorganic polyphosphate. Nat. Mol. Cell. Biol. 58, 71–82
83. Windgassen, M., Sturm, D., Cajiga, I. S., Gonzalez, C. I., Seedorf, M., Bastians, H., and Kreeb, H. (2004) Yeast shutting SR proteins Npl3p, Gsp2p, and Hrb1p are part of the translating mRNAs, and Npl3p can function as a translational repressor. Mol. Cell. Biol. 24, 10479–10491
84. McBride, A. E., Weiss, V. H., Kim, H. K., Hogle, J. M., and Silver, P. A. (2006) The Vtc proteins in vacuole fusion: coupling NSF activity to V(0) trans-complex formation. EMBO J. 25, 259–269
85. McBride, A. E., Cook, J. T., Stemmler Ea Rutledge, K. L., McGrath Ka., O'Neill, E. M., and O'Shea, E. K. (1996) Regulation of PHO4 nuclear localization by the PHO80-PHO85 cyclin-CDK complex. Science 271, 209–212
86. Winter, D. L., Erce, M. A., and Wilkins, M. R. (2014) A web of possibilities: network-based discovery of protein interaction codes. J. Proteome Res. 13, 4869–4897
87. McBride, A. E., Cook, J. T., Stemmler Ea Rutledge, K. L., McGrath Ka., O'Neill, E. M., and O'Shea, E. K. (1996) Regulation of PHO4 nuclear localization by the PHO80-PHO85 cyclin-CDK complex. Science 271, 209–212
88. Winter, D. L., Erce, M. A., and Wilkins, M. R. (2014) A web of possibilities: network-based discovery of protein interaction codes. J. Proteome Res. 13, 5333–5338
89. Albuquerquee, C. P., Smolika, M. B., Payne, S. H., Batina, V., Eng, J., and Zhou, H. (2008) A multidimensional chromatography technology for in-depth phosphoproteome analysis. Mol. Cell. Proteomics 7, 1389–1396
90. Chi, A., Hutterower, C., Geer, L. Y., Coon, J. J., Syka, J. E. P., Bai, D. L., Shabanowitz, J., Burke, D. J., Troyanskaya, O. G., and Hunt, D. F. (2007) Analysis of phosphorylation sites on proteins from Saccharomyces cerevisiae by electron transfer dissociation (ETD) mass spectrometry. Proc. Natl. Acad. Sci. U.S.A. 104, 2193–2198
91. Zappacosta, F., Collingwood, T. S., Huddleston, M. J., and Annan, R. S. (2006) A quantitative results-driven approach to analyzing multisite
protein phosphorylation: the phosphate-dependent phosphorylation profile of the transcription factor Pho4. Mol. Cell. Proteomics 5, 2019–2030

110. Chen, W., Daines, M. O., and Hershey, G. K. K. (2004) Methylation of STAT6 modulates STAT6 phosphorylation, nuclear translocation, and DNA-binding activity. J. Immunol. 172, 6744–6750

111. Yang, J.-H., Chiou, Y.-Y., Fu, S.-L., Shih, I. Y., Weng, T.-H., Lin, W.-J., and Lin, C.-H. (2014) Arginine methylation of hnRNPK negatively modulates apoptosis upon DNA damage through local regulation of phosphorylation. Nucleic Acids Res. 42, 9908–9924

112. Hardiville, S., and Hart, G. W. (2014) Nutrient regulation of signaling, transcription, and cell physiology by O-GlcNAcylation. Cell Metabolism 20, 208–213

113. Kanai, M., Masuda, M., Takaoka, Y., Ikeda, H., Masaki, K., Fujii, T., and Iefuji, H. (2013) Adenosine kinase-deficient mutant of Saccharomyces cerevisiae accumulates S-adenosylmethionine because of an enhanced methionine biosynthesis pathway. Appl. Microbiol. Biotechnol. 97, 1183–1190

114. Albaugh, B. N., Arnold, K. M., and Denu, J. M. (2011) KAT(ching) Metabolism by the Tail: Insight into the links between lysine acetyltransferases and metabolism. ChemBioChem. 12, 290–298