Supplemental Material and Methods

Plasmids construction. Plasmid HTBeaq has been constructed as follows: Plasmid HTB (poly histidine, TEV-protease cleavage site, biotinylation signal) [1] was used as template for a PCR reaction using primers HTBchange_fw: CGG CGG CCG CTA TCC GGT TCT GCT GCT AGG GGT TCA CA TCA T and HTBchange_re: GCG GCC GCC CTC GAG GCC AGA AGA CTC GAC ACT GGA TGG C. The PCR product was reintroduced into plasmid HTB via NotI in order to create new primer annealing sequences. This plasmid was used as a template for two PCR reactions using primer pairs EcoRI-HTB: GCG AAT TCG CGG CCG CTA TCC GGT and HIS-XbaI: GGT TCT AGA CTC TGA TTG AAG TAC CAG and XbaI-BIO: GAG TCT AGA ACC GCC GCA CTA GCT C and HTBchange_re respectively. The PCR products were reintroduced via triple ligation using restriction enzymes EcoRI, XbaI and XhoI (PCR product)/SalI (plasmid backbone) to create an XbaI restriction site. A DNA fragment encoding two additional TEV protease cleavage sites was created by annealing of primers TEV-int-fw: CTA GCG AGA ATC TGT ACT TTC AAG GTC CTG GTG AGA ATC TGT ACT TTC AAG GTG CTT and TEV-int-re: CTA GAA GCA CCT TGA AAG TAC AGA TTC TCA CCA GGA CCT TGA AAG TAC AGA TTC TCG and was subcloned into the XbaI restriction site. This modified HTB plasmid was used as a template for two PCR amplifications using primers 2xHis-fw: GAT CTT AAT TAA GCA TCA TCA CCA CCA TCA TGG TTC ACA TCA TCA CCA CCA TC and annealing-re: CCT CGA GGC CAG AAG AC and primers 2xHis-re: GC TAT TAA TTA ACC TAG CAG CAG AAC CGG ATA GC and p-450-fw: ATA TAT GGA CTT CCA CAC CAA CTA G. The PCR products were individually subcloned and subsequently used for triple ligation using restriction sites SpeI, PacI and XbaI leading to the insertion of a PacI restriction site and an additional 6xHIS sequence prior the original poly HIS sequence of the modified HTB plasmid. The PacI restriction site was used for insertion of a DNA fragment encoding the AQUA (absolute quantification) peptide sequence AADITSLYK obtained by annealing of primers Peptag-fw: TAA AGC TGC TGA TAT CAC TTC TTT GTA TAA AAT and Peptag-re: TTT ATA CAA AGA AGT GAT ATC AGC AGC TTT AAT leading to plasmid HTBeaq-HphMX (pWR168). Marker switch was performed by PCR amplification using plasmid pHTBeaq-HphMX and primers HTBeaqPvuII-LT-fw: GCA TCA GCT GCG GAT CCC CGG GTT AAT TAA TTC CGG TTC TGC TGC TAG and HTBeaq-XhoI-re: GCA TCT CGA GGG AGC CGT AAT TTT TGC TTC GCG CC and plasmid pFA6a-NatMX6 and primers Nat-XhoI-fw-2: ATG CGT CGA CGC GCG CCA GAT CTG TTT AGC TTG CC and Nat-ga-SpeI-re: ATG CAC TAG TCC TCG AGG CCA GAA GAC TCG ATG AAT TCG AGC TCG TTT AAA C to create PCR products HTBeaqNAT1 and HTBeaqNAT2. Both PCR products have been subcloned into PvuII SpeI

cut plasmid pFA6a via triple-ligation using PvuII, XhoI and SpeI restriction sites leading to plasmid pHTBeaq (pWR268).

Yeast strain construction. Yeast strains are listed in Supplemental Table S13. W303 1A SILAC Mat a and Mat α deficient in lysine and arginine synthesis (leu2, ura3, trp1, ade2, arg4, lys1, CAN1), have been obtained as follows: lys1::KanMX and arg4::KanMX cassettes were generated by PCR amplification using primers Arg4-dp-fw: ATC TGC CAA GGC TCC ATC ATA TCT GGC CTA GAA CAG TTT TTT TTT TTA ACG CCA GGG TTT TCC CAG TCA C and Arg4-dp-re: TAT ATA TAT ACA TAG CAC AAT CTC GAA AAT ATA ATA CTA ATA ACA ACC TCA CTA AAG GGA ACA AAA GCT G and Lys1-dp-fw: GCG GCC GCC CTG CGA TTT CAG CGA and Lys1-dp-re: GTT CTT GCT GGG AAA TG and a genomic DNA prep of strain BY 4741 SILAC [2] as a template. The cassettes were transformed into W303 1A Mat A and α respectively and the resulting deletion strains were crossed (and subsequently back-crossed to wt twice) to obtain double knock out strains of both mating types. Additionally, the point mutation in codon 47 of the *can1-100* marker has been reverted to wt to allow the uptake of arginine by transformation of a PCR amplification of the *CAN1* gene from BY 4741 using primers Can1-fw: GTT CTT CAG ACT TCT TAA CTC CTG and Can1-re: ACC TGT ACC AAT AGT ACC ACC AAG. W303 1A SILAC *PAN1*-HTBeaq was obtained by transformation of PCR amplifications of the HTBeaq tagging cassette using plasmid pHTBeaq and primers PAN1-tag-fw: CAG GTA TCC CAT CAA TTC CAC CTG CAG GTA TTC CTC CAC CCC CAC CCC TTC CAT CCG GTT CTG CTG CTA G and PAN1-tag-re: ATA TCA TAT TTG TAA TTC TAC ATC CTT TCA GAA ATT AGT ATA CAT ACG TAT CTC CTC GAG GCC AGA AGA C. *hog1*Δ deletion and *hog1*Δ*kss1*Δ double deletion strains of W303 1A SILAC *PAN1*-HTBeaq were achieved by transformation with deletion cassettes (*HIS3* marker). W303 1A SILAC *PAN1*-HTBeaq *hog1*-as was obtained by a cross with strain W303 *hog1*-as [3].

Measurement of reporter gene expression. Exponentially growing cells harboring the *STL1-lacZ* construct were treated with 0.5M NaCl for 60 minutes or not. β-galactosidase assays were performed as described in [4]. Milligrams of total protein were determined by Bio-Rad Protein Assay (Bio-Rad) according to the manufacturer's protocol.

***In vitro* kinase assay.** *In vitro* kinase assays using purified Pan1-TAP were performed as described elsewhere [3].

SILAC labeling. SILAC labeling is based on methods described elsewhere [5] [2] with the following modifications: SILAC yeast strains expressing HTBeaq tagged Pan1 or not were grown in a preculture until mid log phase in YPD (yeast extract, peptone, D-glucose) media. Cells were harvested by centrifugation (2000xg for 2 mins) and washed twice with SC (synthetic complete) media to remove traces of complete media. The washed cells were used for inoculation of SC supplemented with 0.05 mg/ml of L-arginine:HCl (U-^{13}C6, 97-99%) and 0.05 mg/ml of L-lysine:2HCl (U-^{13}C6, 97-99%) (both Euriso-top) and 0.2mg/ml of proline (Sigma). A second culture containing non-labeled amino acids was inoculated in parallel. Cultures were incubated shaking (180rpm) at 30°C for at least 7 generations until reaching an OD_{600} of 1. In the case of application of osmotic stress 0.5 M NaCl (final concentration) was used for times indicated. Cells were harvested by filtration and immediately deep frozen in liquid N_2.

TRIzol purification. TRIzol purification was performed according to manufacturer's protocol (invitrogen) except: Cells were harvested by filtration and immediately deep frozen in liquid N_2. Cell pellets were resuspended in TRIzol reagent (invitrogen) and cell breakage was performed by bead beating using a FastPrep (3 cycles: 45 secs, Level 6). Glass beads, cell debris and insoluble proteins were removed by centrifugation (12000xg, 15 mins, 4°C). RNA was extracted with addition of chloroform and a subsequent centrifugation (12000xg, 15mins, 4°C). DNA was precipitated by addition of ethanol to inter-phase and organic phase and a subsequent centrifugation (2000xg, 5mins, 4°C). The supernatant was mixed with isopropanol to precipitate proteins. The protein pellet was washed 3 times with 0.3 M Guanidine-HCl in 95% ethanol, 1 time with 95% ethanol, dried in a speed vac and resuspended in 50 mM ammonium bicarbonate (ABC) buffer containing 8 M urea.

Mass spectrometry-based Screen for Hog1 targets. Protein from whole cell extracts (1 mg) was subjected to reduction by dithiothreitol (DTT, 50 µg) at 56°C for 30 min. Reduced cysteine-residues were alkylated with iodoacetamide (IAA, 250 µg) for 30 min at room temperature protected from light. Urea concentration was reduced to 6 M by dilution with 50 mM ABC prior to proteolytic digestion with LysC (Wako Chemicals GmbH; 20 µg) for 2 hours and further diluted to 0.8 M urea for digestion with trypsin (recombinant, proteomics grade, Roche; 50 µg) overnight. After desalting on Strata-X 33 µm Polymeric Sorbent (8B-S100-TAK columns, Phenomenex) and drying, the peptide carboxyl groups were esterified in methanolic HCl as described in [6]. Esterified peptides were dried, re-dissolved in 30% ACN/ 30% methanol/ 40% H_2O and incubated for one hour with 40µl Phos-Select material from

Sigma. After extensive washing with 0.003% acetic acid, peptides bound to the IMAC material were eluted with 50-125 mM Na_2HPO_4 (pH 6.0).

The IMAC-eluates were subjected to nano-HPLC separation on an UltiMate TM 3000 Dual LC System (Dionex, Thermo Fisher Scientific). Peptides were loaded onto a precolumn (PepMAP C18, 0.3 × 5 mm, Dionex) and desalted for 40 min (flow rate 20 µl/min, 0.1% TFA). Peptides were then eluted onto an analytical column (PepMAP C18, 75 µm × 150 mm, Dionex) with a flow rate of 300 nl/min and a gradient from 0% B to 25% B in 120 min and 25% B to 50% B in 60 min, followed by a washing step of 30 min with 90 % B (solvent A: 5% ACN 0.1% formic acid, solvent B: 80% ACN, 0.08% formic acid). The nano-HPLC was coupled online to a hybrid linear ion trap/Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FT, Thermo Fisher Scientific) with a 7-T superconducting magnet via a nano-electrospray ionization source (Proxeon, Thermo Fisher Scientific). Capillary temperature was 200°C and source voltage on metal-coated nano ESI emitters (New Objective) was 1.5 kV. The two detectors of the hybrid instrument were operated in parallel mode: during one full scan in the FT-ICR-cell (resolution 100.000, m/z 400-1800) the five most abundant ions were subjected to MS/MS. Neutral losses of 98, 49 and 32.6 in the MS2 triggered an MS3 analysis in the linear ion trap. Fragmented precursors were excluded from further fragmentation for 60 sec (with 5ppm accuracy) and singly charged peptides were generally excluded from MS/MS analysis. In gas phase fractionation experiments, the full scan mass range was reduced to the following m/z windows: 400-550, 540-700, 690-800, 790-900, 890-1800. Normalized collision energy was set to 35%, activation Q at 25 and activation time at 30 ms.

The obtained spectra were searched by SEQUEST in the Proteome Discoverer 1.3.0.339 software package (Thermo Fisher Scientific) against the SGD database (6717 entries, 03-Feb-2011) plus contaminants. Carbamidomethylation of Cys and methylation of Asp/Glu and the peptide C-terminus were set as static modifications. Phosphorylation of Ser/Thr/Tyr, oxidation of Met and $^{13}C6$ Lys/Arg were set as variable modifications. Tryptic specificity allowing two missed cleavages, a peptide tolerance of 7 ppm and a fragment ions tolerance of 0.8 Da were selected. The automated decoy-search defined the XCorr values versus charge state for a 1% FDR on the peptide level. The probability of phosphorylation site localization was calculated using the phosphoRS software [7] implemented into Proteome Discoverer. Quantification settings were the default values for precursor ions quantifier, the event detection was set to 4 ppm. The result lists were combined in MS Excel and subjected to further data processing: for each SILAC experiment the mixing ratio was determined on the basis of the quantified unique peptides which contained no phosphorylation and no proline. Due to the metabolic Arg-Pro conversion the impact of heavy proline on the H:L ratios was determined: the divergence from a 1:1 ratio of

unphosphorylated peptides containing 1 proline reflected the extent of $^{13}C5$ Pro incorporation and was extrapolated for peptides containing more than 1 proline. For technical and biological replicates the SILAC ratios for identical peptides were averaged. Results are listed in Supplemental Table S1. Annotated MS/MS spectra are available at http://www.proteomecommons.org/tranche under the project name "Yeast PAN1 Phospho" and the password "YIR006C".

MS analysis of SILAC labeled, affinity purified Pan1. Beads were washed five times with ABC buffer (50mM ABC). Disulfide bonds were reduced with DTT (5% w/w of the estimated amount of protein) and Cys-residues subsequently alkylated with IAA (25% w/w of the estimated amount of protein) as described above. DTT (25% w/w of the estimated amount of protein) was added to consume excess IAA and proteins were digested with trypsin (recombinant, proteomics grade, Roche; 5% w/w of the estimated amount of protein) at 37°C overnight. Digests were stopped by addition of TFA to approx. pH 3.

Tryptic peptides were analyzed on an LTQ-FT mass spectrometer (Thermo Fisher Scientific) coupled to the dual-nano HPLC-system as described above except for the HPLC-gradient settings: peptides were eluted from the trapping column onto an analytical column (PepMAP C18, 75 µm × 150 mm, Dionex) with a flow rate of 275 nl/min and a gradient from 0 % B to 100 % B in 90 min, followed by a washing step of 10 min with 10% B and 90% C (solvent A: 5% ACN 0.1 % formic acid, solvent B: 40% ACN, 0.08% formic acid, solvent C: 80% ACN, 10% trifluorethanol, 0.08% formic acid). Mass spectrometer settings were as described above. A full scan (m/z 400-1800, resolution 100.000) was followed by MS2 and neutral loss dependent MS3 analysis of the 5 most intense precursors in the linear ion trap. Peptide identification and SILAC quantification were performed using the SEQUEST algorithm in the Proteome Discoverer 1.3.0.339 software package (Thermo Fisher Scientific). Carbamidomethylation of Cys was set as static modifications. Phosphorylation of Ser/Thr/Tyr, neutral loss of water from Ser/Thr, oxidation of Met and $^{13}C6$ Lys/Arg were set as variable modifications. Spectra were searched against the SGD database (6717 entries, 03-Feb-2011) plus contaminants with tryptic specificity allowing two missed cleavages, a peptide tolerance of 2 ppm, a fragment ions tolerance of 0.5 Da. Quantification settings were the default values for precursor ions quantifier, the event detector was set to 2 ppm. The results were filtered at the Xcorr values to an FDR of 1% on the peptide level. Normalization as well as correction for Arg-Pro conversion was performed in MS Excel as described above (see Supplemental Table S12). The probability of phosphorylation site localization was calculated using the phosphoRS software [7] implemented into Proteome Discoverer. A phosphorylation site probability of 95% or higher was considered as confidently localized. Results were divided in lists of highly (>95%)

and medium (75-95% site probability) confident localized (Supplemental Table S2) and non-localized (< 75% probability) phosphorylation sites (Supplemental Table S3). Annotated MS/MS spectra were exported for all peptide-spectrum matches that mapped to Pan1. This data is available at http://www.proteomecommons.org/tranche under the project name "Yeast PAN1 Phospho" and the password "YIR006C".

iTRAQ analysis. Sample preparation for iTRAQ was as described above, except that 0.5 M triethylammonium bicarbonate (TEAB) was used instead of ABC buffer for washing of the beads and for further sample processing. Reduction and alkylation were performed using 2µL of 50mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) for 1 hour at 60° C and 1µL of 200mM S-methyl-thiomethanesulfonate (MMTS) for 30 min respectively. Trypsin 5% (w/w) of the estimated protein amount was used for overnight digestion at 37°C, however no TFA was added to iTRAQ samples. For iTRAQ labeling, 50µL of ethanol was added to each of the four reagent vials. The unstressed sample was split and labeled with iTRAQ channels 114 and 115, whereas the stressed sample was split and labeled with iTRAQ channels 116 and 117. After two hours at room temperature, equal amounts of channels were mixed, the sample was acidified by addition of TFA and the volume was reduced on a SpeedVac before injection on a U3000 Ultimate nano-HPLC (Dionex). Peptides were loaded onto a precolumn (PepMAP C18, 0.3 × 5 mm, Dionex) and desalted for 15 min with 0.1% TFA as a loading solvent at a flow rate of 25 µl/min. Peptides were then eluted onto an analytical column (PepMAP C18, 75 µm × 150 mm, Dionex) with a flow rate of 275 nl/min using the following ternary gradient: 6.6% B to 51.4% B with 0% C in 180 min, 51.4% B and 0% C to 7.3% B and 90% C in 5 min, maintained for further 5 minutes before going back to 6.6% B and 0% C in 2 minutes, followed by column equilibration for 33 minutes (solvent A: 2.5% ACN 0.1% formic acid, solvent B: 40% ACN, 0.08% formic acid, solvent C: 80% ACN, 0.08% formic acid, 10% trifluoroethanol). The nano-HPLC was coupled online to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) via a nano-electrospray ionization source (Proxeon Biosystems). Data acquisition was started 5 minutes after the LC gradient and switching the precolumn into the column flow. Raw file data acquisition time was 200 min. Capillary temperature was set to 250°C and source voltage was 1.5kV. Lock mass was enabled using the dimethylcyclosiloxane background ions (protonated Si(CH3)2O))6; m/z 445.120025) for internal calibration. The instrument was operated in data-dependent mode. One full scan in the Orbitrap (m/z 350-2000, resolution 60,000, automatic gain control (AGC) target 1E6 ions, maximum fill time 500 ms) was followed by up to 12 data-dependent scans. The four most intense precursor ions were selected for fragmentation by CID, electron-transfer dissociation (ETD) and higher energy collision dissociation (HCD) excluding singly charged ions. Selected precursor masses were subsequently excluded from fragmentation for 180 s

using an exclusion window of +/- 5 ppm. In order to fragment peptides close to the apex of the elution profile, chromatography mode was enabled, using an expected peak width of 10 s and the correlation option with a maximum area ratio to the precious scan of 0.8. For CID scans, an AGC target of 1E4 ions and a maximum fill time of 200 ms were used, isolation width was set to 2.4 Da, and normalized collision energy was 35% with an activation time of 10 ms. Multi-stage activation was activated for CID scans with a neutral-loss mass list of 32.6, 49 and 98 Th. For ETD spectra, the AGC target was set to 1E4 ions with a maximum fill time of 200 ms, the isolation width was 3 Da, and the normalized collision energy was 35%, with an activation time of 100 ms and supplemental activation. Reagent ion target was 1E5 ions with a maximum fill time of 80 ms. HCD scans were recorded at a resolution of 7,500 in the Orbitrap, with an AGC target of 1E5 ions and a maximum fill time of 250 ms. For HCD the isolation window was narrowed to 1.6 Da in order to reduce the likelihood of co-isolation and fragmentation of ions with similar m/z, a known source for the compression of iTRAQ ratios towards unity. In order to allow both, identification and quantification from HCD scans, stepped collision energy with two steps HCD 42% and 58% was used. All MS/MS spectra were searched against the database for identification, however quantification was based on reporter ions extracted exclusively from HCD spectra. Discoverer 1.3.0.339 was used for database search and iTRAQ quantification. For each iTRAQ reporter ion, the area of the ion with the m/z closest to the theoretical m/z within a 5 mmu window was extracted from the raw file. Only scans in which all four reporter ions were detected were used for quantification. Reporter ion areas were corrected for isotope impurity, and ratios were calculated from corrected values. Spectra were searched with SEQUEST against the SGD database (6717 entries) supplemented with a set of common contaminant sequences and concatenated with the reversed version of each sequence. The searched FASTA-file contained 13834 sequences. iTRAQ-modification of Lys and the peptide N-terminus, as well as Methylthio-Cys were set as static modifications. Variable modifications were oxidation of Met, deamidation of Gln and Asn, iTRAQ modification of Tyr, and phosphorylation of Ser, Thr and Tyr. Precursor mass tolerance was 20 ppm, and fragment ion tolerances were 0.5 Da for CID and ETD spectra and 50 mmu for HCD spectra. b and y ions (and neutral loss ions) were used for scoring spectra in CID and HCD searches, whereas c and z ions were used for ETD searches. Scans were filtered as follows: Search engine rank 1, peptide length min 8 amino acids, 7 ppm, and a SEQUEST peptide probability of 50% or higher (3.01) for CID and HCD spectra (no probability was available for ETD spectra). In addition, for each type of search result, an XCorr cutoff was chosen that led to a FDR below 1% at the spectrum level (CID: 2.06, ETD: 3.08, HCD: 1.03). For each spectrum, the geometric mean of ratios 116:114 and 117:115 was calculated. Peptides identified in different MS/MS spectra were considered only once when the HCD spectrum used for quantification was the same. For peptides quantified by several HCD spectra, a

geometric mean was calculated. Phosphorylation site localization was calculated using the phosphoRS software [7]. Results are listed in Supplemental Tables S1 and S2 according to the site probability. Peptide-Spectrum Matches assigned to PAN1 containing both phosphopeptides and non-phosphopeptides as well as quantitative data are listed in Supplemental Table S7. Annotated MS/MS spectra were exported for all peptide-spectrum matches that mapped to Pan1. This data is available at http://www.proteomecommons.org/tranche under the project name "Yeast PAN1 Phospho" and the password "YIR006C".

SRM analysis. The peptide mixture was separated on a reversed phase nano-HPLC (Ultimate 3000, Dionex). Peptides were concentrated and washed with 0.1% TFA on a trapping column (PepMap C18, 300µm×5mm, 3µm, 100Å) for 30 min at a flow rate of 25 µl/min. Bound peptides were eluted and separated on an analytical column (PepMap C18, 75µm×150mm, 3µm, 100Å) using a linear gradient from 2.5% to 40% ACN with 0.1% formic acid in 60 min at a flow rate of 300 nl/min. The HPLC was directly coupled to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific) via a nano-electrospray ion source (Proxeon). The Q1 peak width of the instrument was set to 0.7 u. Synthetic peptides were used to select the most intense transitions and the optimal collision energies and to determine the retention time for the peptides of interest. An SRM method with 56 transitions (50ms dwell time each) containing transitions for the peptides of interest in phosphorylated and unphosphorylated form, as well as for 3 additional, unmodified peptides of Pan1 and the quantification peptide in the tag for normalization purposes was set up. The Pinpoint 1.0 software (Thermo Fisher Scientific) was used for data analysis. For the timecourse experiments, a scheduled SRM method was set up with a cycle time of 2 seconds and the same list of 56 transitions. For a list of used transitions/CEs, see Supplemental Figure 2C. For SRM quantitation experiments with unpurified Pan1 whole cell extracts were prepared with Trizol as described above. Phosphopeptide enrichment with titanium dioxide (TiO2) using phtalic acid was performed as described in Bodenmiller et al. 2007 [8]. Fold over 0 minutes values of peak areas of reference peptide transitions (see Supplemental Figure 2C: AQUA, Ref-1, Ref-2, Ref-3) were calculated for each time point. The values were averaged and used for determination of loading differences (normalization factor). Fold over 0 minutes values of peak areas of transitions were individually averaged and corrected with the normalization factor to determine induction values from peptides of interest (T 1225, T#1225, S 1003, S#1003) for each technical replicate. Induction values of the technical replicates were averaged for each peptide which resulted in the final fold over 0 minutes ratio. Induction values of the individual technical replicates were also used to determine geometric standard

deviation. Results were multiplied with/divided by the geometric standard deviation to get the confidence interval (see Supplemental Tables S7, S8, S9 and S10).

Supplemental Results

A tandem affinity purification tag for quantitative MS studies.

We reconstructed and modified the original HB-tag (poly histidine, biotinylation signal) [1] to improve efficiency and versatility of the tandem affinity purification protocol. We named the new tag HTB-eaq (effective cleavage plus absolute quantification: see below and Supplemental Figure 1A). Our modifications include a stretch of 12 histidines (poly-His) instead of six as well as three optimal TEV protease cleavage sites (instead of one suboptimal site) allowing effective TEV cleavage of the tag if required, respectively. Moreover, we included a peptide sequence AADITSLYK for absolute quantification for SRM measurements as described in Wepf *et al* [9]. Finally, we expanded the flanking sequences to allow PCR amplification of the cassettes according to Longtine *et al* [10] or, alternatively, to Knop *et al* [11].

We optimized the purification protocols allowing time-saving one-step purifications as well as high purity tandem affinity purification to efficiently analyze proteins of different abundance (Supplemental Figure 1B). As a test of the efficiency of the purification methods we compared protein pull downs of endogenously expressed Pan1-HTBeaq using either one-step or tandem purification protocols. Supplemental Figure 1C shows an overlay of the UV chromatograms of trypsin digests of the differently purified samples. A one step purification using Ni^{2+} sepharose led to samples of high peptide complexity and therefore high impurity. However, a one-step purification using the biotin-streptavidin affinity led to samples of significantly reduced complexity that were suitable for quantitative MS analysis of low abundance peptides. Highest sample purity was achieved when a tandem affinity purification protocol was applied.

We also tested how the different purification protocols would perform in a phosphopeptide SRM analysis using a modified peptide of Pan1 (SSS#PSYSQFK). While samples solely purified with Ni^{2+} sepharose gave a high number of background peaks that led to a wrong automated selection for quantification, the one-step purification protocol using streptavidin agarose provided samples of higher purity that could already be sufficient for the analysis of low intensity signals (Supplemental Figure 1D). Tandem affinity purification led to an even further reduction of the background peaks and is therefore the method of choice for experiments in which synthetic peptides are unavailable or in which the number of diagnostic fragments is limited.

Comparison of different strategies for sample purification for SRM analysis.

Since we obtained consistent results using SILAC, iTRAQ and SRM (Fig 3D) we wanted to establish a standard

sample preparation procedure for SRM analysis to monitor the kinetics of Pan1 phosphorylations in response to hyperosmotic stress. We analyzed phosphopeptides SSS#PSYSQFK and SVHAAVT#PAAGK and the corresponding unmodified peptides from samples obtained by TiO_2 phosphopeptide enrichment of whole cell extracts (WCE) and also by different purification protocols such as one step purification protocols using either solely Ni^{2+} sepharose or streptavidin agarose as well as the tandem affinity purification (Supplemental Information and Supplemental Figure 1). SRM measurements of samples derived from TiO_2 enrichment of WCE were unsatisfying, because transition signals of the phosphopeptides were close to background and transition peaks of the corresponding unphosphorylated peptides could not clearly be assigned (Supplemental Figure 1E and Supplemental Table S9). However, we obtained reproducible results when purified Pan1 was analyzed. We were able to observe a consistent up/down-regulation of peptides in response to hyperosmotic stress that were also comparable to our previous results. Ratios for the low abundance peptide SVHAAVT#PAAGK showed the highest variation, probably due to the low basal level.

Pan1 is an in vitro substrate of MAP kinase Hog1.

We tested for the ability of the MAPK Hog1 to directly phosphorylate the endocytotic protein Pan1 *in vitro*. Supplemental Figure 2B shows that a purified Hog1 analogue sensitive (as) kinase allele was able to phosphorylate tandem affinity (TAP) purified Pan1. Phosphorylation signals were significantly reduced when the kinase was inhibited by an ATP analogue (SPP86) prior to the kinase reaction. These data strongly support a direct connection of the kinase Hog1 and the endocytotic pathway.

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Supplemental Table Legend

Supplemental Table S1: Identified phosphopeptides from the SILAC shotgun experiment. In two biological SILAC replicates yeast cells were stressed for 5 min with high osmolarity. SILAC2 was measured with three different methods. Unique phosphopeptides identified in these runs are listed in a combined list with the averaged ratio L/H. Cells are marked in blue if the peptide was identified in the corresponding run. SEQUEST-modification, XCorr, number of missed cleavages, Δppm, pRS probability and H/L ratio of all unique phosphopeptides are listed for each experiment separately.

Supplemental Table S2: Confidently localized phosphorylation sites of Pan1. Pan1-phosphopeptides from all experiments (SILAC and iTRAQ) are listed. List is divided into phosphopeptides with a phosphorylation site probability of >95% and of 75% - 95% according to PhosphoRS 2.0.

Supplemental Table S3: Non localized phosphorylation sites of Pan1. Pan1-phosphopeptides from all experiments (SILAC and iTRAQ) with a phosphorylation site probability of <75% are listed.

Supplemental Table S4: SILAC quantification of Pan1 peptides in wild type. Search results from 4 biological replicates are listed. Shown are the averages of normalized stress induction ratios.

Supplemental Table S5: SILAC quantification of Pan1 peptides in *hog1*Δ. Search results from 3 biological replicates are listed. Shown are the averages of normalized stress induction ratios.

Supplemental Table S6: SILAC quantification of Pan1 peptides in *hog1*Δ *kss1*Δ. Search results from 2 biological replicates are listed. Shown are the averages of normalized stress induction ratios.

Supplemental Table S7: iTRAQ quantification of Pan1 peptides in wild type. Search result from a single run. Shown are the averages of normalized stress induction ratios.

Supplemental Table S8: Comparison of quantitative MS strategies: SRM data. Peak areas of transitions (listed in Supplemental Figure 2C) for phosphorylation sites S1003 and T1225 have been determined for the light and heavy labeled peptides. Stress induction ratios (blue) have been normalized with AQUA-peptide (factor indicated in green) and corrected Arg-Pro conversion (red).

Supplemental Table S9: Sample preparation strategies for SRM. Peak areas of transitions (listed in Supplemental Figure 2C) for S1003 and T1225 are listed. Stress induction ratios (blue) have been normalized with AQUA-peptide and three reference peptides of Pan1 (factor indicated in green) except for WCE TiO_2 samples. Transitions of reference peptides from WCE TiO_2 are indicated on the right and labeled with "$" and "$$".

Supplemental Table S10: Phosphorylation kinetics by SRM measurements. Peak areas of transitions (listed in Supplemental Figure 2C) for S1003 and T1225 are listed. Stress induction ratios (blue) have been normalized with AQUA-peptide and three reference peptides of Pan1 (factor indicated in green). Time point 0 min has been set to 1.

Supplemental Table S11: SRM measurements for Hog1-inhibitor studies. Peak areas of transitions (listed in Supplemental Figure 2C) for S1003 and T1225 are listed. Stress induction ratios (blue) have been normalized with AQUA-peptide and three reference peptides of Pan1 (factor indicated in green). Time point 0 min has been set to 1.

Supplemental Table S12: Normalization and correction factors for Arg-Pro conversion. Normalization factors were calculated for each biological sample based on unique peptides containing no phosphorylation and no proline. The correction factors for Arg-Pro-conversion was determined by the H:L ratio of unphosphorylated peptides containing 1 proline and were extrapolated for peptides containing more than 1 proline.

Supplemental Table S13: Strains and plasmids used in this study.

Supplemental Figure Legend

Supplemental Figure 1:

Performance of the HTBeaq-tag. A: HTBeaq contains a quantification peptide sequence (AQUA), one additional 6xHis tag and two additional TEV cleavage sites. Modifications are highlighted by red boxes. The 158 amino acid sequence is suitable for tagging yeast proteins under the control of the endogenous promoter. B: Optimized protein purification protocol that allows tandem affinity purification of proteins under denaturing conditions. Alternatively a one-step purification using STRP-Agarose as the sole affinity resin can be performed. Purified proteins can be directly digested from the affinity resins and phosphopeptides can be further enriched via TiO_2 if necessary before the final analysis by reverse phase nano-HPLC-MS. C: Comparison of HPLC UV chromatograms (λ=214nm) of either His tag purified (blue), biotinylation tag purified (red), or tandem purified (green) Pan1-HTBeaq. RT: retention time. D: SRM transitions of phosphopeptide SSS#PSYSQFK of Pan1 (S1003) obtained from the three differently purified Pan1-HTBeaq samples. Singly and doubly charged C-terminal y-ions are indicated. NL: neutral loss. Peaks automatically selected for quantification by the software are marked with “Area”. Inset on the left shows SRM transitions of the Ni^{2+}-Sepharose purified sample in higher resolution. Actual peak of peptide SSS#PSYSQFK is marked with an arrow. Inset on the right shows SRM transitions obtained with a synthetic peptide. E: Comparison of different strategies for sample preparation for SRM. Ratios (stressed/unstressed) obtained for phosphopeptides SSS#PSYSQFK (S1003) or SVHAAVT#PAAGK (T1225) and the corresponding unphosphorylated peptides are indicated. SRM measurements have been corrected for proline conversation (correction value was obtained from SILAC quantification Supplemental Table S12).

Supplemental Figure 2:

SRM analysis of Pan1 phosphopeptides. A: SRM transitions of phosphopeptides SVHAAVT#PAAGK (T#1225) and SSS#PSYSQFK (S#1003) and of corresponding unphosphorylated peptides shown in Figure 5B. SRM transitions of the quantification peptide embedded in the HTBeaq tag is labeled as AQUA. Pan1-HTBeaq samples were obtained by affinity purification from cultures that have been treated with 0.5 M NaCl for times indicated. Singly and doubly charged C-terminal y-ions are indicated. NL: loss of phosphoric acid. WL: loss of water. B: Patterns and retention times of SRM transitions of peptides obtained from affinity purified Pan1-HTBeaq fit those of corresponding synthetic peptides. SRM transition peaks of a representative sample (sample: wild type + DMSO (mock), osmo-stressed for 10 minutes) are shown. Peaks automatically selected for quantification by the software

are marked with “Area”. C: Detailed list of peptides used in our SRM analysis. m/z values of precursor and singly and doubly charged C-terminal y-ions are indicated. CE: collision energy used for fragmentation. Synthetic peptides were used to select diagnostic transitions and the optimal collision energies and to determine the retention time for the peptides of interest. For reference peptides Ref-1, Ref-2 and Ref-3 diagnostic transitions were selected from CID spectra.

Supplemental Figure 3:

The kinases Prk1 and Hog1 phosphorylate Pan1. A: The phosphorylation status of the Prk1 consensus ([L/I/V/M]xx[Q/N/T/S]xTG) site T 570 of Pan1 remains unaltered in response to osmotic stress. SILAC labeled Pan1-HTBeaq was purified from wt, *hog1*Δ strain backgrounds. Untreated cultures were labeled with ^{13}C arginine and ^{13}C lysine; osmo-stressed (10 minutes 0.5 M NaCl) cultures with ^{12}C arginine and ^{12}C lysine. Phosphothreonine 570 has been previously identified as a target site of kinase Prk1 [12]. We obtained a 1:1 ratio for phosphopeptide T#GFGNNEIYTK in all experiments. B: *In vitro* kinase assay using Hog1-as and yeast purified TAP-tagged Pan1 as a substrate. Kinase reactions were done for 30 minutes in the presence (+) or absence (-) of 5µM of as-inhibitor SSP86.