Absolute Proteome and Phosphoproteome Dynamics during the Cell Cycle of Schizosaccharomyces pombe (Fission Yeast)*

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To quantify cell cycle-dependent fluctuations on a proteome-wide scale, we performed integrative analysis of the proteome and phosphoproteome during the four major phases of the cell cycle in Schizosaccharomyces pombe. In highly synchronized cells, we identified 3753 proteins and 3682 phosphorylation events and relatively quantified 65% of the data across all phases. Quantitative changes during the cell cycle were infrequent and weak in the proteome but prominent in the phosphoproteome. Protein phosphorylation peaked in mitosis, where the median phosphorylation site occupancy was 44%, about 2-fold higher than in other phases. We measured copy numbers of 3178 proteins, which together with phosphorylation site stoichiometry enabled us to estimate the absolute amount of protein-bound phosphate, as well as its change across the cell cycle. Our results indicate that 23% of the average intracellular ATP is utilized by protein kinases to phosphorylate their substrates to drive regulatory processes during cell division. Accordingly, we observe that phosphate transporters and phosphate-metabolizing enzymes are phosphorylated and therefore likely to be regulated in mitosis. *Molecular & Cellular Proteomics* 13: 10.1074/mcp.M113.035824, 1925–1936, 2014.

Cell replication involves a complex series of highly regulated and evolutionary conserved events, called the “cell cycle.” Aberrations in the cell cycle have severe implications and can cause cancerous growth. A detailed understanding of the cell cycle and its regulation may identify additional targets for cancer therapy (1–3). The cell cycle has been the subject of previous proteomics studies. Olsen *et al.* (4) measured the dynamics of thousands of proteins and phosphorylation events across cell cycle phases of HeLa cells, providing insights into the underlying regulatory mechanisms and pointing to a general increase in phosphorylation site occupancy during M phase. In a targeted study, Pagliuca *et al.* (5) investigated interactors of cyclins E1, A2, and B1 in HeLa cells, revealing key mechanistic links between DNA replication and mitosis.

Schizosaccharomyces pombe (fission yeast) is a unicellular organism, which can easily be genetically manipulated and carries many cell cycle features similar to metazoan cells. It is an important model organism to study the cell cycle and its checkpoint controls (6). Recent global proteomics studies of yeasts and their cell cycle (7–13) have mainly focused on Saccharomyces cerevisiae (budding yeast), with only a few studies of fission yeast (14, 15), although the fission yeast cell cycle may be more representative of eukaryotic cell cycles (16). However, attention of the proteomics community toward S. pombe is increasing. Recent proteomics studies covered up to 4087 S. pombe proteins (71% of the predicted proteome) and 1544 phosphoproteins in both asynchronous and synchronized cell cultures (17–22); however, a comprehensive analysis of the S. pombe cell cycle is so far missing.

Here, we use high resolution mass spectrometry in combination with stable isotope labeling by amino acids in the cell culture (SILAC)1 method, termed super-SILAC (23), and intensity-based absolute quantification (iBAQ) (24) to measure relative and absolute dynamics of the proteome and phosphoproteome during the cell cycle of fission yeast. We estimate copy numbers for 3178 S. pombe proteins, and we combine these data with calculated phosphorylation site stoichiometry to estimate the total amount of protein-bound phosphate and its dynamics across the cell cycle. Providing the global absolute dynamics and stoichiometry of proteins and their modifications will be a valuable resource for classical and systems biologists alike.

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1 The abbreviations used are: SILAC, stable isotope labeling by amino acids in cell culture; iBAQ, intensity-based absolute quantification; IN2, liquid nitrogen; P-event, phosphorylation event; RT, room temperature; SPE, solid phase extraction; SAX, strong anion exchange chromatography; SCX, strong cation exchange chromatography; TiO2, titanium dioxide; FDR, false discovery rate.
Cell Cycle Proteome Dynamics of *S. pombe*

EXPERIMENTAL PROCEDURES

**Overview of the Experimental Design**—SILAC-labeled cells were synchronized using the temperature-sensitive cdc25-22 allele (25). Briefly, cells were grown at 25 °C (permissive temperature) to a cell density of 6–7 × 10^6^ cells/ml and arrested at late G2 phase by shifting to 36 °C (restrictive temperature) for 5 h. Cells were released into the cell cycle by shifting the temperature back to 25 °C. The Lys-0-labeled cells were harvested at 17 min (M phase) and 32 min (G1 phase), whereas the Lys-8-labeled cells were harvested at 0 min (G2 phase; without shifting back to 25 °C) and 50 min (S phase). To perform relative quantification of the four cell cycle phases, we generated a super-SILAC standard (23) by mixing an equal number of cells from the G2, M, G1, and S phases labeled with medium-heavy lysine (Lys-4). We added this standard to equal numbers of cells from M (Lys-0) and S (Lys-8) phases and separately from G1 (Lys-0) and G2 (Lys-8) phases, resulting in two triple-SILAC samples (Fig. 1). Each sample was digested with endoproteinase Lys-C followed by fractionation via isoelectric focusing (Off-Cell) and strong anion exchange chromatography (SAX). Phosphopeptides were enriched using strong cation exchange chromatography (SCX) followed by titanium dioxide (TiO2) chromatography. The resulting fractions were measured on LTQ-Orbitrap Elite and LTQ-Orbitrap XL mass spectrometers and a 10-ml cell culture were pelleted to prepare a trichloroacetic acid (TCA) protein extract that was analyzed by immunoblotting.

**Cell Harvesting**—We harvested 1.26 × 10^9^ cells at each cell cycle phase. Cells were pelleted by centrifugation (3000 × g, 2 min, centrifuged at 40 °C for G2 samples or 4 °C for samples from other phases). All tubes and reagents were kept at 37 °C for G2 samples and at 4 °C for samples from other phases. After washing with H2O and decanting of the supernatant, a small amount of residual liquid remained, in which cells were resuspended. The cell suspension was dripped into liquid nitrogen (lN2) so that cells were shock-frozen and maintained in denaturation buffer (6 M urea, 2 M thiourea, 1% (w/v) n-octylglucoside (Roche Applied Science), 10 m mw Tris, pH 8.0) to a final protein concentration of −10 mg/ml. The suspension was incubated for about 1 h at 4 °C while shaking before insoluble material was removed twice by centrifugation (7197 × g, 10 min, 4 °C). The supernatant was aliquoted and stored at −80 °C. This procedure yielded −130 and 60 mg of protein for the SILAC standard and M/S and G2/G1 samples, respectively.

**Microscopy**—Methanol-fixed cells were washed with PEM/methanol (100 mM PIPES, 1 mM EGTA, 1 mM MgSO4, pH 6.8, with KOH), 50% methanol and with PEM. For DNA staining, cells were resuspended in 100 μl of PEM containing 2 μl/mg DAPI (Sigma) and incubated at room temperature for at least 10 min. Cells were pelleted, and 4 μl of the pellet were mounted. Images were acquired on an Axio Imager.M1 microscope (Zeiss) with a Plan-Apochromat 63x/1.4 oil objective (Zeiss) driven by a Piezo 2 motor. Images were recorded with a charged-coupled device camera (CoolSnap EZ, Roper) and processed using the software MetaMorph 7.6.0.0 (Molecular Devices). 14 individual planes spaced by 0.3 μm were acquired. To create color-combined pictures, out-of-focus planes were removed; 10 sections of each the GFP and mCherry channels were projected (maximum intensity projection), and signal intensity was adjusted for all pictures in a similar way. For the DAPI channel, only one of the in-focus planes was chosen for the color-combined picture.

**Immunoblots**—Proteins were separated by SDS-PAGE and transferred onto PVDF membranes (Immobilon-P, Millipore) using a semi-dry transfer system (Amersham Biosciences). Primary antibodies are listed in supplemental Table 1. Horseradish peroxidase (HRP)-coupled secondary antibodies were detected by chemiluminescence.

**Trichloroacetic Acid Extraction**—4 × 10^7^ cells were harvested by centrifugation (3000 × g, 1 min, RT) and resuspended in 1 ml of cold 20% (v/v) TCA. Cells were pelleted by centrifugation (775 × g, 2 min, RT). The supernatant was removed, and cells were washed with 1 ml of 1 m Tris and pelleted again. The cell pellet was resuspended in 100–200 μl of 2× SDS sample buffer (125 μl Tris, pH 6.8, 4% (v/v) SDS, 20% glycerol, 200 μm DTT, 0.02% (w/v) bromphenol blue) and boiled at 95 °C for 10 min. 600 μl of glass beads (500 μm) were added, and the samples were vortexed strongly (FastPrepTM120 BIO101, Thermo Electron Corp.; three times for 40 s, level 5, with a 3-min break in between the individual runs). A hole was made in the bottom of the tube with a needle, and the cell extract was eluted in a new reaction tube by centrifugation (60 s, 95×g, 4 °C) thereby separating the extract from the glass beads. The resulting samples were boiled again at 95 °C for 10 min and centrifuged (16,000 × g, 10 min, RT) to pellet the cell debris.

**Intracellular ATP Quantification**—Cells were disrupted using a ball mill grinder (Mixer Mill MM 400, Retsch). ATP was extracted using 5% (v/v) TCA and measured using the ENLITEN kit (Promega) according to the manufacturer’s instructions. After ATP extraction, samples were neutralized with 20 mM sodium acetate buffer and diluted 50- and 1000-fold with ATP-free water. The luciferase reagent was added, and luminescence was recorded for 10 s after a 2.5-s wait. A standard curve, using ATP included in the kit, was used to transform relative luminescence units into ATP concentration. The amount of cells measured in each sample was used to calculate ATP molecules per cell. Because the cdc25-22-synchronized cells have a cell volume that is 3.51 times larger than unsynchronized cells (27), the resulting value was divided by 3.51.

**In-solution Digestion**—The SILAC standard was added to the G2/G1 and M/S samples before digestion. For absolute quantification, the value was divided by 3.51. Because the cdc25-22-synchronized cells have a cell volume that is 3.51 times larger than unsynchronized cells (27), the resulting value was divided by 3.51.
diluted with 4 volumes of water before digestion overnight at RT with 1:100 (w/w) endoproteinase Lys-C or 1:100 (w/w) trypsin (low urea concentration). The protein digest was acidified using TFA to a final concentration of 0.1% (v/v) to stop the protein digestion. In the case of samples fractionated using the Off-Gel fractionation, no TFA was added.

**Phosphopeptide Enrichment**—After protein digestion, 7.5 mg of the samples was enriched for phosphopeptides using SCX followed by TiO$_2$ chromatography as described before (29) with minor modifications. Samples were injected using SCX Buffer A (5 mM KH$_2$PO$_4$, 30% acetonitrile (ACN), pH 2.7) and separated with a gradient from 0 to 35% of SCX Buffer B (350 mM KCl, 5 mM KH$_2$PO$_4$, 30% ACN, pH 2.7). The resulting 15 fractions and phosphopeptides from the flow-through were further enriched by TiO$_2$ chromatography. Three consecutive rounds of enrichment were performed with the flow-through. The resulting 18 samples were eluted three times with 40% ammonium hydroxide solution in ACN with pH 10.5. After elution, samples were evaporated to a final volume of 5 μl and injected into the LC-MS system.

**Off-gel Fractionation**—100 μg of protein digest were fractionated by isoelectric focusing using the OFFGEL 3100 Fractionator (Agilent) according to the manufacturer’s instructions (30). Separation was performed on a 13-cm Immobiline DryStrip (GE Healthcare) at a maximum of 50 μA for 20 kV-h. The peptide fractions were purified using Stage-Tips (31).

**SAX**—50 μg of protein digest were fractionated using SAX (32). Briefly, digested proteins were desalted using solid phase extraction, mixed 1:1 with Britton and Robinson Universal Buffer at pH 11 (0.02 M CH$_3$COOH, 0.02 M H$_3$PO$_4$, and 0.02 M H$_2$BO$_3$), and loaded onto a Stage-Tip-based anion exchange column containing six layers of Empore anion exchange (Varian). The flow through was collected and desalted using a Stage-Tip. Peptides were then eluted with Britton and Robinson Universal Buffer at pH 8, 6, 5, 4, and 3 and desalted using Stage-Tips.

**Nano-LC-MS/MS**—An Easy-nLC II system (Proxeon Biosystems) coupled to an LTQ Orbitrap XL, an LTQ Orbitrap Velos, or an LTQ Orbitrap Elite (Thermo) was used for the LC-MS/MS measurements. Peptides were separated on a 15-cm PicoTip fused silica emitter inner diameter of 75 μm (New Objective) packed in-house using reversed-phase beads ReproSil-Pur C18-AQ 3 μm (Dr. Maisch, GmbH). Peptides were injected with Solvent A (0.5% acetic acid) at a maximum pressure of 280 bar using the IntelliFlow option and separated at 200 nl/min. The separation was performed using a linear 106-min gradient of 5–33% solvent B (80% ACN in 0.5% acetic acid) for phosphopeptide samples and either an 81- or a 221-min segmented 5–50% solvent B gradient for all other samples.

For proteome samples, an LTQ Orbitrap Velos and an LTQ Orbitrap Elite were operated in positive mode. Precursor ions were recorded in the Orbitrap analyzer at a resolution of 60,000 (LTQ Orbitrap Velos) or 120,000 (LTQ Orbitrap Elite) followed by MS/MS spectra acquired in the LTQ mass analyzer. Data-dependent analysis was used, and either the 15 (LTQ Orbitrap Velos) or the 20 (LTQ Orbitrap Elite) most abundant precursor ions from the full scan were fragmented using collision-induced dissociation (CID). Fragmented masses were excluded for 90 s. The target values were 1E6 charges for the MS scans in the Orbitrap and 5000 charges for the MS/MS scans in the LTQ with a maximum fill time of 100 and 25 ms, respectively.

Phosphoproteome samples were measured using an LTQ Orbitrap XL with the same parameters as for the proteome samples with the following changes: precursor ions were recorded at 60,000; the five most abundant precursor ions were fragmented by activation of the neutral loss ions at –98, –49, and –32.6 Thompson relative to the precursor ion (33); the maximum fill time was 150 ms for MS and 1000 ms for MS/MS scans.

**Data Processing and Analysis**—MS raw files were processed using MaxQuant software (Version 1.2.2.9) (34). Multiplicity was set to three, matching the number of SILAC labels used ("light," “medium,” and “heavy”) in each experiment; Lys-4 and Lys-8 were specified as medium and heavy labels, respectively. Database search was performed after peptide quantification using the Andromeda search engine (35), which is part of MaxQuant. Full enzyme specificity was required, and up to two missed cleavages were allowed. For proteome and phosphoproteome measurements, Lys-C was specified as the enzyme used, and for lysine acetylation-enriched samples, trypsin was specified as the enzyme used. The MS/MS spectra were searched against a target database consisting of 5,075 entries from the PomBase S. pombe proteome database, 50 entries from the Proteomics Dynamic Range Standard (UPS2, Sigma), the mCherry protein sequence, the enhance GFP protein sequence, and 248 frequently seen laboratory contaminants. To control the false discovery rate of database search results, the software appends a corresponding decoy version of the target database resulting in a total of 10,750 database entries. Carbamidomethylation on cysteines was defined as fixed modification, and oxidation of methionine and protein N-terminal acetylation were set as variable modifications. Initial mass tolerance for the precursor ion was set to 6 ppm, and fragment ion mass tolerance for CID fragmentation data was set to 0.5 Da. For HCD fragmentation data, the fragment ion mass tolerance was set to 20 ppm. In addition, we included phosphorylation of serine, threonine, and tyrosine (STY) and acetylation of lysine as variable modifications for the data derived from samples enriched for phosphopeptides and lysine-acetylated peptides, respectively. Database search results were further processed for statistical validation, and identified MS/MS spectra were assembled into peptides, protein groups, and modification sites at a default false discovery rate (FDR) of 1% at all levels. The estimated FDR after database search was 1% at the protein group level, 0.97% at the phosphorylation site level, and 0.54% at the peptide level (S. pombe and UPS proteins). A list containing S. pombe peptide and UPS sequences detected at an estimated FDR of 0.54% without reverse hits and without contaminants can be found in supplemental Table 2. For protein quantification, a minimum of two quantitation events was required. Peptide evidences of single-peptide-based protein identifications are listed in supplemental Table 3.

**Relative Quantification**—The two triple SILAC experiments were combined to get a ratio profile for each protein and phosphorylation event across the four cell cycle phases relative to the Lys-4-labeled super-SILAC standard. Ratios of proteins and phosphorylation events were transformed to log$_2$ scale, and only proteins and phosphorylation events (P-events) that were quantified in all four phases were considered for further quantitative analysis. The magnitude of fluctuation was expressed by calculating the standard deviation of the log-transformed ratios across the four phases. The quartiles of the distribution resulting from all quantified features were determined and used to separately bin proteins and P-events according to the extent of fluctuation across the cell cycle phases. Those belonging to the quartile with the highest standard deviation (75–100%) were defined as “fluctuating” or dynamic, whereas those belonging to the quartile with the lowest standard deviation (0–25%) were defined as “nonfluctuating” or static.

**Absolute Protein Quantification**—We used the iBAQ approach for absolute protein quantification, which calculated the absolute protein amounts proportional to the molar amounts of an added protein standard consisting of 48 proteins (UPS2, Sigma). Resulting iBAQ values were subsequently adjusted according to the amount of UPS2 protein that was added to one of the measured samples (M and S cell cycle phases, Off-gel proteome measurement). The total number of cells in the sample (N$_{cells}$) was used to calculate the absolute protein copy numbers (CN$_{protein}$) by applying Equation 1.

\[
CN_{protein} = \frac{iBAQ_{protein}}{N_{cells} \times 10^{-12}}
\]
with \( N_p \) being the Avogadro constant. Because the cdc25-22-synchronized cells have a cell volume that is 3.51 times larger than unsynchronized cells (27), the resulting values were divided by 3.51 to obtain the copy number in a typical unsynchronized cell. This assumes that protein concentration is similar between cdc25+ and cdc25-22 cells, which is not formally known. The absolute protein amounts calculated for the super-SILAC standard together with corresponding SILAC ratios were used to calculate the absolute amount for each protein in the four cell cycle phases.

**Absolute Phosphorylation Site Occupancy**—The proportion between the phosphorylated and nonphosphorylated version of the same peptide defines the occupancy of the corresponding phosphorylation site. Olsen et al. (4) described a model to calculate the occupancy of singly phosphorylated peptides by using the SILAC ratios of the following: 1) the phosphorylated peptide; 2) the nonphosphorylated peptide; and 3) the corresponding protein, assuming that all three ratios are measured accurately, that the ratios change between the different states, and that there are only two versions of the peptide (nonphosphorylated and phosphorylated on a single site). For details on the calculation see Olsen et al. (4). We calculated occupancy values separately for the two replicates of our dataset using an in-house developed perl script. The SILAC ratios of phosphorylated peptides were derived from the corresponding SCX fractions, whereas for nonphosphorylated peptides and proteins, we used the SILAC ratios measured in the proteome measurements (Off-gel).

**Absolute Phosphate Stoichiometry**—We calculated the absolute phosphate stoichiometry as a product of site occupancy and protein copy number of the protein carrying the phosphorylation site. This calculation was done only when both values for a specific phase were available (101 sites in G2 phase, 71 in sites M phase, 81 in G1 phase, and 67 in S phase). To estimate the global level of protein-bound phosphate, we first inferred estimates of median occupancy and standard deviation by performing 10,000 bootstrap iterations on the available occupancy values in each cell cycle phase. We then replaced missing occupancy values with values obtained by sampling from a normal distribution using the bootstrap estimates as parameters for mean and standard deviation and calculated absolute phosphate stoichiometry as described above. We calculated bootstrap confidence intervals for the median occupancy using the 0.025 and 0.975 percentiles of the bootstrap distribution resulting in a 95% confidence interval.

**Peak Time Index Calculation**—We performed the analysis on expression peak time indices of proteins and phosphorylation sites as described previously (4). Briefly, the SILAC ratios for each item (i.e. protein or P-event) were normalized to the maximal change across the four cell cycle phases. For each item, we calculated the weighted mean of the expression ratio at the phase with maximal change with respect to the normalized ratios of adjacent phases. To assign the resulting “peak time index” of every item to a specific cell cycle phase, we applied hierarchical clustering on these values using the Euclidian distance and a defined cluster number corresponding to the four cell cycle phases addressed in this study.

**Functional Enrichment Analysis**—We retrieved Gene Ontology annotation of S. pombe from GeneDB (downloaded on December 6, 2011). To test whether specific annotation terms are enriched or depleted within a set of proteins of interest, we applied Fisher’s exact test using the theoretical S. pombe proteome as background. Derived \( p \) values were further adjusted to address multiple hypothesis testing using the method proposed by Benjamini and Hochberg (36).

**RESULTS**

We measured relative and absolute dynamics of the proteome and phosphoproteome during the four major phases of the cell cycle, G1, M, G2, and S, using highly synchronized S. pombe cultures and SILAC (Fig. 1 and supplemental Fig. 1). To relatively quantify all four cell cycle phases, we generated a super-SILAC standard (23), consisting of a mixture of cells from all four phases. The standard was combined with two of the cell cycle phase-specific samples (either G1 or G2 or M and S) to yield a triple-SILAC sample. The quantitative information from both triple-SILAC measurements was integrated through this common standard to obtain the relative dynamics of the proteome and phosphoproteome across the cell cycle (Fig. 2A). Fluorescence microscopy confirmed more than 90% synchronicity for all analyzed cell cycle phases, and characteristic changes were verified by immunoblotting (Fig. 2, B and C, and supplemental Fig. 1). In 137 LC-MS runs, we recorded 2,471,241 MS/MS spectra that identified a total of 32,016 nonredundant peptides from 3753 S. pombe proteins at estimated false discovery rates (FDR) of 0.53% at the peptide and 0.99% at the protein level. The identified proteins accounted for 74% of the predicted S. pombe proteome (5075 proteins). In the phosphoproteome measurement, we identified 3682 phosphorylation events (on 1554 proteins) of which 2465 were localized to a specific Ser/Thr/Tyr residue (supplemental Table 4). The identified and quantified proteins as well as phosphorylation events are presented in supplemental Tables 5 and 6. Additionally, we detected 328 acetylation events on 184 proteins (including histones, cohesin, SAGA subunits, ribosomal proteins, and metabolic enzymes; see supplemental Table 7), which were not analyzed further due to technical bias toward highly abundant proteins as a result of low enrichment efficiency (data not shown).

**Relative Quantification of Proteome and Phosphoproteome Dynamics**—Application of the super-SILAC approach enabled relative quantification of 2416 proteins and 1963 phosphorylation events across all analyzed phases of the cell cycle. We achieved high reproducibility between the added-in super-SILAC standard of each triple SILAC sample (0.9 ≤ \( r \) ≤ 0.93) (supplemental Fig. 2). Changes on the protein level were minimal (Fig. 3A), which lead to a relatively low correlation of protein ratios (0.26 ≤ \( r \) ≤ 0.62) (supplemental Fig. 3A). In contrast, changes at the phosphoproteome level were considerably more pronounced (Fig. 3B) and showed high reproducibility between both replicates (0.7 ≤ \( r \) ≤ 0.82) (supplemental Fig. 3B). Normalization of phosphorylation site abundance to the protein level resulted in negligible differences (supplemental Fig. 3, C and D), so we analyzed phosphorylation data without protein normalization. We validated our data by performing immunoblots and fluorescence microscopy to measure dynamics of selected positive controls, which were overall in agreement with the MS data (supplemental Figs. 4 and 5).
To assess the extent of fluctuation of proteins and phosphorylation events across the cell cycle, we used the standard deviation of log-transformed SILAC ratios across the different cell cycle phases and binned the proteins and phosphorylation events into quartiles of the resulting distribution (Fig. 3, C and D). Proteins or phosphorylation events with the highest standard deviation were defined as fluctuating. This analysis confirmed that strong fluctuation predominantly occurred at the phosphoproteome level (1032 fluctuating events, see supplemental Table 8), whereas protein abundance during the cell cycle was much more static (only 54 fluctuating proteins were detected, see supplemental Table 9). Of these 1032 fluctuating phosphorylation events and 54 proteins, 638 events and 37 proteins were quantified across all four analyzed cell cycle phases in the replicate analysis. From this overlap, 591 phosphorylation events were fluctuating in both replicates and showed high correlation in the relative expression across the four cell cycle phases (median correlation coefficient, 0.99). A lower number of proteins (10) was fluctuating in both replicates and had a lower correlation (median correlation coefficient, 0.41), likely as a consequence of the lower extent of protein fluctuation (Fig. 3, E and F). Relative expression of fluctuating proteins and phosphorylation events can be found in supplemental Data Files I and II. Because of high correlation, especially of fluctuating phosphorylation events, we concluded that each of the replicates is representative of the whole dataset, and therefore we proceeded with one replicate for further analysis.

**Functional Classes of Fluctuating Proteins and Phosphorylation Events**—The strongest fluctuating protein that was identified in all four phases was the cyclin B Cdc13 (high in G2 and M and low in G1 and S, supplemental Table 9), whose degradation at the transition from M to G1 is well known (Fig. 2C) (37). Strongly fluctuating was also the septation protein Uds1 (highest in S phase). This had been observed by MS previously (38), and we confirmed this by immunoblotting and fluorescence microscopy (Fig. 2C and supplemental Figs. 4 and 5). On the phosphoproteome level, we recovered the well known cell cycle-regulated phosphorylation of histone H3-Ser-10 (39) (Fig. 2C), as well as several previously known cell cycle-specific phosphorylations, including mitosis-specific phosphorylation of monopolin Mde4 (40), survivin Bir1 (41), and previously identified sites phosphorylated by Aurora kinase (17). Interestingly, we observed considerable cell cycle-specific fluctuation in the phosphorylation of nucleoporins, which mimics observation in human cells (42), despite the fact that fission yeast, unlike human cells, undergoes a closed mitosis (Fig. 4A and supplemental Table 8). We performed functional enrichment analysis for the proteome and the phosphorylation data separately for the different quartiles (supplemental Fig. 6, A and B). As expected, cell cycle-related domains and processes such as regulation of cyclin-dependent protein kinase activity and septum were over-represented among the fluctuating proteins. House-
Fig. 2. Data integration and synchronicity for all analyzed cell cycle phases. A, samples are compared with each other using a super-SILAC standard (STD) containing a mixture of G2, M, G1, and S phases. By comparing the information of these two experiments through the common standard, a profile for each quantified protein and phosphorylation event is obtained. B, synchronization of the cells was confirmed by fluorescence microscopy using Plo1-mCherry (M phase) and enhanced GFP-proliferating cell nuclear antigen (EGFP-PCNA) validating our approach (supplemental Fig. 6, B). Functional enrichment analysis of proteins and phosphorylation events in the standard and experimental samples. MPM2 is an antibody generated against mitosis-specific phosphopeptides (supplemental Table 1).

Keeping processes like the TCA, citric acid and urea cycles, glycolysis, ribosome, and translation were over-represented among nonfluuctuating proteins. At the phosphorylation level, cell cycle cytokinesis, mitotic cell cycle spindle assembly checkpoint, spindle assembly, and regulation of transcription during mitosis were over-represented, whereas translation and mitochondrial translation were among the under-represented functional classes. Our global analysis also indicated which kinases have a large influence on cell cycle-dependent fluctuations (CDK1, CDK2, and Polo kinase) and which kinases act mostly in a cell cycle-independent fashion (CK1 and CK2) (supplemental Fig. 6C).

For each of the fluctuating proteins or phosphorylation events, we determined the cell cycle phase with the highest value and used the peak time indices (4) as a similarity measure for hierarchical cluster analysis. The resulting four clusters contained the proteins and phosphorylation events that were most prominent in each analyzed cell cycle phase (Fig. 4, D and E). Functional enrichment analysis of proteins and phosphorylation events in the resulting phase-specific clusters revealed a difference in kinase-related functions increased specifically in the G2 phase. Furthermore, several mitosis-related biological processes were increased during mitosis, further validating our approach (supplemental Fig. 6, D and E).

Using the measured SILAC ratios of phosphoproteins, phosphopeptides, and their unmodified counterparts, we calculated stoichiometry (occupancy) of 101 phosphorylation sites in G2 phase, 71 sites in M phase, 82 sites in G1 phase, and 67 sites in S phase. In agreement with previous studies (4), the highest median occupancy of 44.2% was measured in M phase. Average occupancy was reduced in the subsequent phases reaching a minimum of 23% in G2 phase (Fig. 5).

Absolute Quantification—We estimated absolute levels of S. pombe proteins using the iBAQ approach (24). This method correlates the MS signal intensities and the number of observable peptides of each protein to an added-in protein standard. We added 48 proteins from 0.5 to 50,000 fmol into one fraction of our measurements (Off-gel fractions of M and S phase triple SILAC measurement) and detected 23 of the added-in proteins at concentrations between 50 and 50,000 fmol. This pointed to an under-representation of very low abundant proteins. The detected standard proteins enabled the calculation of iBAQ values for 3,178 S. pombe proteins (supplemental Fig. 7A).

The dynamic range of protein copy numbers spanned almost 5 orders of magnitude, which was in agreement to the dynamic range determined in asynchronously growing S. pombe cells (Fig. 6A and supplemental Table 10 and supplemental Fig. 8) (18, 22). The total number of protein molecules in an average S. pombe cell was 176 million, with single proteins having between 92 (Snf59) and 1.4 million (enolase) copies. Eighty percent of the whole protein content was constituted by 20% of the most abundant proteins, consistent with the Pareto 80–20 principle, also observed by Marguerat et al. (18) (Fig. 6B).

We compared our absolute quantification to several studies that used different technologies to derive absolute protein amounts in S. pombe as follows: MS intensity-based absolute quantification (18, 19, 22) and fluorescence microscopy-based quantitation (27, 43). It has been reported that...
cdc25-22 cells under the used growth conditions are 3.51-fold larger, which was taken into account when normalizing the protein copy numbers (see under “Experimental Procedures”) (27). Overall, we found a good correlation of protein copy numbers compared with previous studies (Pearson correlation > 0.7, see supplemental Fig. 7, B–F). Our numbers of proteins and phosphorylation events across the cell cycle phases. Diagrams for each quantified protein group (A) and phosphorylation event (B) relative to the super-SILAC standard plotted against its intensity recorded in the mass spectrometer. Red dots indicate statistically significant (p < 0.01) outliers from the distribution. The box plots below the figures depict the distribution of SILAC ratios described above. Box plot diagrams of standard deviations (S.D.) were calculated from SILAC ratios across the four phases of the cell cycle for proteins (C) and phosphorylation events (D); dashed lines represent the quartiles of standard deviations based on the whole dataset; these were used to group proteins and phosphorylation events according to their extent of fluctuation across the cell cycle; the upper quartile (>75%) was used to define fluctuating proteins and phosphorylation events (see “Experimental Procedures”). Overlap of fluctuating proteins (E) or fluctuating phosphorylation events (F) between the replicates and box plot of correlation coefficients between the profiles of fluctuating proteins or phosphorylation events.
Cell Cycle Proteome Dynamics of *S. pombe*

**FIG. 4.** Selected cell cycle-dependent phosphorylation events and sorting of protein and phosphorylation variation according to peak time index. Cell cycle-dependent variation in the phosphorylation of nuclear pore or nuclear membrane-related proteins (A), inorganic phosphate transporters (B), or phosphate metabolism enzymes (C) is shown. Each circle represents a phosphorylation event detected on the respective protein and is colored according to the cell cycle phase with the highest phosphorylation; circles with a P represent phosphate. Proteins (D) or phosphorylation events (E) within the quartile of highest fluctuation were clustered in the cell cycle phases according to their peak time index (see “Experimental Procedures”). Protein and phosphorylation events in this figure can be found and are highlighted in supplemental Tables 8 and 9.

**FIG. 5.** Phosphorylation site stoichiometry. A, box plots of phosphorylation site stoichiometry calculations for each cell cycle phase (see “Experimental Procedures”). The number of phosphorylation sites is shown at the top of the figure, and the numbers in the boxes represent the median values of all calculated phosphorylation site occupancies. B, cumulative diagram of the phosphorylation site occupancy in the studied cell cycle phases.

were higher when compared with Gunaratne et al. (19); however, they were in agreement with the other four studies used for comparison (18, 22, 27, 43). The stoichiometry of several known stable protein complexes was also compared, and subunit stoichiometry was in agreement with previous studies (Table I). All studies dealing with estimation of absolute pro-
Estimation of the Absolute Amount of Protein-bound Phosphate Groups—The availability of phosphorylation site occupancies and absolute protein copy numbers enabled us to address the absolute amount of Ser/Thr/Tyr phosphorylation in the different phases of the cell cycle. To estimate the absolute level of protein-bound phosphate, we added the missing occupancy values by bootstrapping (see under “Experimental Procedures”) and calculated the number of protein-bound phosphate for all proteins that we absolutely quantified. Our results showed that the total number of protein-bound phosphate groups in each cell cycle phase (see “Experimental Procedures”).

The availability of phosphorylation site occupancies and absolute protein copy numbers enabled us to address phosphorylation during the G2/M transition. Consistently, we observed phosphorylation (and hence presumably regulation) of phosphate transporters and enzymes of phosphate metabolism during mitosis (Fig. 4, B and C, and supplemental Tables 6 and 9).

DISCUSSION

The cell cycle is a complex dynamic process involving multiple layers of regulation, and its global analysis is challenging. The super-SILAC approach combined with high accuracy mass spectrometry is a powerful tool that enables integrative analysis of several samples by comparing them with a common standard. Super-SILAC was used previously to analyze specific proteomes of various cell lines (46), cancer
Cell Cycle Proteome Dynamics of *S. pombe*

The use of SILAC-based relative quantification enabled us to address the stoichiometry of detected phosphorylation events. As expected, the overall phosphorylation level was peaking in mitosis, where it was about 2-fold higher than in the other cell cycle phases. Our median occupancy of single sites in M phase (44%) was significantly lower than reported in HeLa cells (~70%) (4). The reasons could include biological (species-related) differences as well as experimental differences, because nocodazole was used to arrest HeLa cells, whereas our cells progressed through mitosis unperturbed.

It is important to point out that calculations of absolute levels depend on many different parameters such as cell counting, cell size measurement, iBAQ measurement, determination of UPS standard, and protein concentration. As each of these parameters presents a source of error, only approximate absolute values can be determined. Nevertheless, our dataset shows good correlation with previously reported similar datasets and satisfactory internal control parameters, such as stoichiometry of known complex subunits. Hence, the data reported here will be a useful resource for classical and systems biologists alike.

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REFERENCES


